INFLUENCE OF CELL ENVIRONMENT ON MICRONUCLEATION IN CHINESE HAMSTER OVARY CELLS

A Dissertation

by

NATALIA GENNADIEVNA MEDVEDEVA

Submitted to the Office of Graduate Studies of
Texas A&M University
in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

August 2004

Major Subject: Nuclear Engineering

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Approved as to style and content by:

John R. Ford (Chair of Committee)	Leslie A. Braby (Member)
John W. Poston (Member)	Robert C. Burghardt (Member)
	William E. Burchill (Head of Department)

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ABSTRACT

Influence of Cell Environment on Micronucleation in

Chinese Hamster Ovary Cells. (August 2004)

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Chair of Advisory Committee: Dr. John R. Ford

The irradiation of cells in culture is an essential part of many radiation biology experiments. Since these experiments necessarily involve the irradiation of cell culture vessels and nutrient medium, the possibility of effects due to the interactions of irradiated material with growing cells needed to be investigated.

In the present study the micronucleus frequency in Chinese hamster ovary (CHO) cells as a function of such parameters as type of radiation, type of cell substrate, changes in cell environment, and time course of the effect were characterized. Observations of the persistence of micronucleus formation in irradiated CHO cells reveal that the number of cells containing micronuclei reaches its maximum within nine hours after irradiation and remain elevated for at least five days. The influence of the cell environment on micronucleus formation in CHO cells was examined by plating cells in preirradiated nutrient medium or on preirradiated cell culture vessels. In all experiments, pre-irradiation of the cell substrate (the culture dish or culture dish filled with medium) led to a significantly higher micronucleus frequency than when cells were plated on un-irradiated substrate. The difference is most pronounced at the lowest doses examined.

These results suggest that methods of cell culture vessel sterilization and the composition of cell attachment surfaces could be confounding factors, particularly in the experiments which are intended to examine the response of cells exposed to low doses of ionizing radiation.

DEDICATION

To my daughter Alexandra who always makes sure that my life is not easy… but worth living.

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CHAPTER I

INTRODUCTION

Ionizing radiation is a physical agent known to induce chromosome aberrations, mutations and to promote cancer. However, ionizing radiation has gained great importance in the treatment of different types of cancer. Therefore, the study of cell and tissue responses to ionizing radiation is an important task in both radiation biology and oncology. The persistence of radiation-induced chromosomal lesions (micronuclei) is a major asset when assessing risk, since persistent lesions may be accumulated in the target tissue. Therefore, the micronucleus formation in Chinese hamster ovary cells under the different conditions, the behavior of micronuclei *in vitro* and their influence on the fate of cells exposed to ionizing radiation is the area of interest for the present study.

Ionizing radiation, as many other cytotoxic agents, may induce whole chromosomes or chromosome fragments that do not attach properly to the spindle apparatus during mitosis. These chromosomes or fragments then may be spontaneously enclosed by nuclear membrane, resulting in a micronucleus. This micronucleus is separated from the two nuclei but remains in the cytoplasm of one of daughter cells after completion of cytokinesis. Micronuclei do not arise only from whole chromosomes that lag at mitosis due to a damaged kinetochore or to a faulty mitotic apparatus.

This dissertation follows the style and format of *Radiation Research.*

They can also arise from acentric fragments that fail to be incorporated into the daughter nuclei during cell division because of the lack of a kinetochore, or from complex chromosomal rearrangements that encounter mechanical difficulties during anaphase.

Micronucleus formation may occur at any subsequent stage of mitosis after prophase. When the lagging chromosome is a nucleolus-forming chromosome, then the resulting micronucleus will exhibit a nucleolus. Fundamental questions remain concerning the mechanisms by which micronuclei originate, the chromosomes from which they are derived, and the significance of their manifestation to health outcomes.

Experimental data suggest that DNA replication and RNA synthesis can take place in micronuclei as in the main nucleus (1). In addition, micronuclei can undergo mitosis. Considering that micronuclei no longer remain under the normal cell control, it is often supposed that they are lost after successive cellular divisions. Some groups suggest that considerable number of micronuclei may be masked by daughter nuclei and may not be counted (2). Some others believe that micronuclei may get reincorporated into a nucleus (3). It was found (1) that in some cases after prophase rearrangement of the cell the micronucleus membrane dissolves and the chromosomes which formed the micronucleus form its own metaphase plate which later merges with the metaphase plate of the main nucleus. This suggests that sometimes during the micronucleus life a functional activity of kinetochores could be restored. However, few studies of micronucleus persistence have been performed and it is still unclear whether the persistence is different when dealing with micronuclei showing a centromere signal (i.e. a micronucleus containing a whole chromosome), or micronuclei harboring acentric

fragments. Micronucleus persistence is an especially important factor when a genotoxicity study is performed in highly proliferative cell systems such as bone marrow cells. There are indications that both lagging whole chromosomes and acentric fragments participate in the initial radiation-related increase in the frequency of micronuclei, confirming the ability of ionizing radiation to induce not only chromosome breakage, but also aneuploidy. Micronuclei containing chromatin without centromeres were found to be as unstable as acentric fragments and dicentrics (4). Chang, *et al*. found that acute high-dose radiation exposure generate higher proportions of acentric fragments included in micronuclei than whole lagging chromosomes (5).

There is also a hypothesis (6) that micronuclei form around unrepareble DNA loops, leading to accumulation of DNA fragments. Measurements of double-strand breaks at twenty four hours after irradiation showed that the increased terminal deoxynucleotidyl transferase (TdT) labelling in the observed micronuclei occurred concomitantly with a reduced TdT label in the nucleus, suggesting that nuclear DNA fragments are incorporated into micronuclei. Also it was found that the transcriptional activity existing in micronuclei was comparable with that of the nucleus in almost half of the micronuclei. The authors (4, 6) suggested that micronucleus formation may represent another way to deal economically with incomplete repair of damaged cells. Since cells that cannot be repaired completely may not have to be replaced immediately, the process of micronucleation seems to be plausible as an efficient way of retaining of damaged cells temporarily.

Elevated micronucleus frequency in irradiated cells may also be caused by an altered mitotic apparatus. Sato, *et al*. hypothesized that increased micronucleus formation in cells exposed to ionizing radiation may partly be due to centrosome dysfunction. They found that irradiated cells with abnormal numbers of centromeres frequently exhibited striking defects in morphology of the nucleus, including micronucleation. Quantitative analysis revealed a significant correlation between multiple centrosome formation and nuclear abnormalities in irradiated cells. These findings imply that radiation-induced damage and/or nuclear abnormalities, especially the formation of nuclear fragments, may be attributed to the spindle defects resulting from multiple centromeres (7).

Ponsa, *et al*. in their study of chromosome loss in gamma-irradiated human lymphocytes found that the type of micronuclei formed by cells depends on the dose of ionizing radiation. Based on those observations the following hypotheses were developed:

1) At low doses micronuclei contain whole chromosomes in addition to acentric fragments, while at high doses there is a greater likelihood for two or more aberrations being included in a single micronuclear membrane or being retained in the main nucleus so that they do not produce individual micronuclei.

2) Ionizing radiation, although principally clastogenic, also has aneuploidogenic properties.

3) There is engulfment of chromosomal material that otherwise might form micronuclei at the end of mitosis (8).

The phenomenon known as the dose-rate effect has the great influence on micronucleus formation. From a wide range of radiobiological studies (primarily conducted at the cellular level), dose rate effects are observable in the range of 1 - 100 cGy/min. In this interval, the biological effect of a given total dose is decreased as the dose rate is decreased (presumably due to biological repair of radiation damage). Dose rates below 1 cGy/min do not reducce the biological response significantly. Similarly, above 100 cGy/minute, biological responses are not increased significantly. The dose-rate effectiveness factor (DDREF) has been used to describe this reduced response at low dose rates compared to high rates. Therefore, that the dose-rate must be considered when evaluating radiation risks, as numerous studies have reported a significantly reduced response with decreased dose-rates. Chronic exposures to Cs^{137} at three dose-rates were shown to induce significant increase in the frequency of translocations and erythrocyte micronuclei in mice (9). Micronucleus responses for both micronucleated polychromatic erythrocytes and micronucleated normochromatic erythrocytes were found to be significantly above background and dependent upon daily dose-rate, but were higher for micronucleated polychromatic erythrocytes than for micronucleated normochromatic erythrocytes. Gender differences were found in baseline levels of both micronucleated polychromatic erythrocytes and micronucleated normochromatic erythrocytes, with females being less sensitive than males. A significant increase of translocations per 100 cell equivalents was found after acute expose compared to chronic and fractionated exposures. Dose-rate reduction effects were found to range from 3 at 50 cGy to 14 at 350 cGy for acute compared to chronic exposures and

1.9 for fractionated compared to chronic exposures which is in line with DDREF for ICRP 60 (10).

Dose-rate effects for apoptosis and micronucleus formation induced by Cobalt-60 gamma-rays in human lymphocytes were studied by Boreham, *et al*. They found that there was a very small intra- and interindividual variation between donors for micronuclei formation. In all donors there was up to two-fold decrease in micronucleus formation when the dose rate of the 4 Gy dose was decreased from 70 cGy/min to 0.29 cGy/min. They also found that the apoptotic index showed dose-rate dependence at a dose-rate lower than that for the micronucleus formation. They suggested that the mechanisms or signals for processing radiation-induced lesions for these two end-points must be different. There appear to be two mechanisms which contribute to the dose-rate effect for micronucleus formation: one independent on cell-cycle delay and one dependent on a cell-cycle delay induced only at very low dose rates, the same low dose rates which showed a reduction in apoptosis. Since the dose-rate at which cells showed reduced apoptosis as well as a further reduction in micronucleus formation was very low, they concluded that the processing of the radiation lesions that induce apoptosis, and some micronuclei, is very slow (11).

The micronucleus frequency in cells exposed to ionizing radiation depends not only on dose-rate, but also on linear energy transfer (LET). High-LET radiations such as neutrons and alpha particles are more destructive to biological material than low-LET radiations, such as X rays and gamma rays, because at the same dose, the low-LET radiations produce the same number of ionizations more sparsely within a cell, whereas

the high-LET radiation transfers most of their energy to a small region of the cell. The localized DNA damage caused by dense ionizations from high-LET radiations is more difficult to repair than the diffuse DNA damage caused by the sparse ionizations from low-LET radiations. So, exposure of cells to high-LET radiation results in a higher micronucleus frequency than exposure to low-LET radiation (12 – 15).

In the study of Sgura, *et al*., the frequency of aberrant chromosome segregation in human fibroblasts after irradiation with low-energy protons of different energies and after X-ray-irradiation was determined by using fluorescent dye techniques. The results have shown that micronucleus induction was dependent on the linear energy transfer and that the majority of micronuclei contain only chromosome fragments. X rays, despite having a decreased ability to induce the micronuclei compared to low energy protons, produced a higher frequency of micronuclei containing both centromeres and kinetochores. These micronuclei are indicative of the integrity of the centromeric region and they suggest that DNA represents a negligible target for the induction of X-ray-induced aneuploidy. They hypothesized that high energy released along the tracks of the protons leads to "partial" aneuploidy as a result of clastogenic effect at the centromeric region. X rays, which induced noncentromeric DNA damage, were found to able to induce chromosome loss through some mechanisms involving non-DNA targets (12).

Studying V79 cells exposed to non-targeted alpha-particles and low energy protons, Prise, *et al*. found that in high-LET irradiated cells death occurs by other mechanisms not involving the formation of micronuclei. Using a microbeam to deliver individual protons to the center of the cell nucleus, they found that a single 3.2 MeV

proton delivering around 0.02 Gy to the nucleus of a V79 cell gave a 1% probability of producing a micronucleus. The yield of micronucleated cells was a linear function of the number of particles delivered and the distribution of micronuclei in irradiated cells followed a Poisson distribution for cells exposed to less than thirty protons (13).

It has been shown that micronucleus frequency in rat alveolar epithelial cells was much higher when cells were exposed to alpha particles compared to exposure of the cells to X rays. The relative biological effectiveness (RBE) of alpha particles as calculated from the dose-response curves was found to be 4.3 (14).

Since micronucleus formation reflects the influence of ionizing radiation on different cells in dose-dependent and LET-dependent manner, measurement of micronuclei frequency is gaining importance as a technique for estimating radiation dose. The other reasons for this choice are the rapidness and ease of the micronucleus test, good statistical power and the possibility of automation. In addition, the micronuclei test is suitable for detection of clastogens and aneugens due to the fact that chromosome fragments and whole chromosomes may be included in micronuclei. Therefore, micronucleus tests are currently utilized in cancer therapy to investigate cancer cell response to treatment as well as for evaluation of ionizing radiation exposure consequences in cases of radiation accidents. The majority of researchers tend to use the micronucleus assay introduced many years ago by Fenech and Morley (15). This technique utilizes cytochalasin B for arresting cytokinesis. The use of cytochalasin B facilitates scoring of micronuclei in the first division post-exposure by inhibiting cytokinesis and thus minimizing any loss of the chromosomal fragments from

subsequent cell divisions. This method is often referred to as cytokinesis-blocked micronucleus assay.

The results presented in the study of Ramirez, *et al*. clearly reflect the strong requirement of cell division for micronucleus expression and the high instability of micronuclei during further cell divisions. A rapid increase of micronuclei was observed on cell division after irradiation, reaching a peak at three days after irradiation. All of the induced micronuclei disappeared within one week after irradiation, which is approximately three cell cycles after the initial induction, when the frequency of micronuclei declined to background levels (4).

Micronucleus frequency in human peripheral blood lymphocytes has been used as an indicator of chromosome damage for over 20 years. Peace, *et al*. used this test in combination with physical removal of micronuclei by microdissection to examine if the formation of micronuclei is operated by a non-random process. In conclusion they proposed the screening for chromosome changes utilizing micronucleus assay with aim to predict development of cancer and other diseases, particularly those related with aging. They suggest that this screening is also immediately applicable to the processes such as the accumulation of genetic damage with aging, the interaction of environment and individual susceptibility, and the interaction of lifestyle factors and disease development (16).

Gutierrez, *et al*. used a micronucleus assay to assess possible chromosome damage induced by I^{131} therapeutic exposure. In their study the frequency of both binucleated cells with micronuclei and the total number of micronuclei in cultured

peripheral blood lymphocytes of treated patients was analyzed. They concluded that the age, as source of variation, appears to be one of the most important confounding factors in the analysis of micronuclei in blood lymphocytes when conducting biomonitoring studies. In general, the results of their study support the idea that the micronucleus analysis in peripheral lymphocytes in hyperthyroidism and thyroid cancer patients treated with I^{131} is sensitive enough to detect possible genotoxic effects associated with therapeutic exposure to radioiodine and that these effects can persist for at least 1 year after therapy (17).

Those results are in agreement with the data obtained by Chang, *et al*., which indicates that most lymphocytes carrying micronuclei or chromosomal aberration are slowly turned over in the body circulation over years. The estimated half-life of micronuclei in human individuals exposed to gamma-radiation was around 37.2 months (5).

Jagetia, *et al*. suggest that the majority of the human cancers are caused by tobacco smoke and natural and synthetic chemicals of occupational, environmental, medical and dietary origin and use the micronucleus assay to determine the genotoxic and mutagenic potentials of the mentioned above agents in the peripheral blood lymphocytes of cancer patients (18). The peripheral blood lymphocytes seemed to be suitable for such type of studies because of their easy availability, widespread distribution, synchronous population, low frequency of spontaneous chromosomal aberrations, convenient culture methods and ease sample collection. However, they did not find any correlation of micronuclei frequency in the cultured peripheral blood

lymphocytes and the age and sex of patients. What they found was a significant elevation in the frequency of micronucleated binucleated lymphocytes, which correlated with the habits of smoking, tobacco and pan chewing and/or alcohol consumption of the patients. The research group suggests that the regular use of these genotoxic agents might have resulted in the development of neoplastic disorders in these patients. The elevation of the micronucleated binucleated cells during the middle and at the end of the radiation treatment may be helpful in evaluation of the response of the patients towards the treatment. Thus, they concluded that periodical analysis of micronucleated binucleated cells in the lymphocytes of patients may also help to assess the tumor response to therapy.

Recently, the phenomenon known as the bystander effect has been the subject of numerous studies (19-24) extensively reviewed by Morgan (20, 21). This phenomenon has triggered an avalanche of speculation about its possible mechanisms and its impact on risk assessments. Since most of bystander experiments involve cell irradiation *in vitro*, there may be many factors that may affect the experimental results. One of these factors may be the environment surrounding the cells. The objective of our study is the characterization of micronucleus formation in Chinese hamster ovary cells as a function of such parameters as type of radiation, type of cell substrate, time-course and using a micronucleus assay as a tool to assess the influence of pre-irradiated cell culture vessels and medium on cells seeded on those culture vessels. Evaluation of the effect of the altered environmental factors due to irradiation is essential to properly evaluating the

risks due to radiation exposure whether it is due to environmental, medical or occupational sources.

CHAPTER II

PHYSICAL CHARACTERISTICS OF MICRONUCLEATION

Introduction

Biological dosimeters are essential tools needed to obtain information concerning the distribution and extent of radiation exposures after radiation accidents or radiation experiments. A bio-dosimeter has a further advantage in that an individual's damage is measured. Micronucleus formation is an endpoint that is widely used for monitoring and evaluation of the clastogenic effects of chemicals and radiation in a range of cell-types (25-29). The micronucleus assay has been routinely used as a biological dosimeter (26, 30-32) and can be a useful tool to evaluate cell sensitivity to ionizing radiation with implications for tumor therapy (33, 34). Simple and fast, this method can be successfully used for processing of a large series of samples over a short period of time. However, there are some shortcomings associated with this method. The data of *in vivo* micronucleus assays are hard to interpret due to variations in individual response to ionizing radiation and varying micronucleus background frequencies, which may be influenced by such factors as environment, lifestyle, diet, and many others (31, 32, 34, 35). Also, a standard protocol for sample preparation and micronucleus scoring has not been adopted for all cell types. Therefore, it is difficult to compare data from different research groups.

In addition, some other complications may arise with the micronucleus assay due to impact of such factors as bystander effects (19, 20, 36, 37), low dose hypersensitivity (21), adaptive response (21, 38, 39) and sample storage time (40) on the micronucleus frequency. Therefore, the first step in this study was to identify a protocol which would be most applicable to the Chinese hamster ovary cell line (CHO-K1). A slightly modified micronucleus assay was utilized to study the sizes of nuclei and micronuclei in CHO cells and quantify the relationship between the size of the nucleus in the cell and intensity of fluorescent signal from 6-Diamidino-2-PhenylIndole (DAPI)-stained cells. DAPI was chosen for DNA staining in cells because of its ease of handling, visualization and resistance to photo bleaching. Also using DAPI allows easy verification in the absence of some cell-culture contaminants such as Mycoplasma (41).

Materials and methods

Cell culture

Chinese Hamster Ovary (CHO) cells from American Type Culture Collection were maintained in F-12 Nutrient Mixture (Ham) solution (Gibco BRL) with 10% Fetal Bovine Serum (Hy Clone) at 37 ^{0}C in a humidified atmosphere containing 5% CO_2. Cells were propagated in 75 cm^2 tissue-culture flasks (Costar) and passed at confluence to Mylar®-bottomed dishes or glass-bottomed tissue-culture containers for radiation experiments.

Irradiation procedure

Irradiation was carried out under room temperature at exposure rate 1 Gy/min. Cm^{244} disk source (diameter 10 mm, activity 10 μCi at 3/1/98, activity was 8.7 μCi at the time of the experiment) was used for alpha-irradiation.

X-ray-irradiation was carried out using 250 kVp X rays from a Norelco X ray machine.

Each experiment was repeated at least two times with three parallel samples for each point. The evaluation of the significance of the data was performed using one-tailed Student's t-test with α=0.05.

Cytokinesis-block method

Cytokinesis-blocking was performed as suggested by M. Fenech and A. Morley (42). A stock solution of cytochalasin B at 1 mg/ml was prepared in 100% ethanol. Prior to the experiment an aliquot of the stock solution was mixed with medium, so that the final concentration of cytochalasin B was 3 μg/ml. Three hours after incubation at 37^{0}C

in this medium, cells were rinsed with PBS, treated with 0.075 M KCl for seven minutes, fixed with 70% methanol and stained with 1.5 μg/ml DAPI.

Scoring of micronuclei

Micronuclei were scored using a Zeiss Axiovert 200 M microscope equipped with appropriate DAPI filters. At least 150 binucleated cells were scored for micronuclei on each sample. The criteria for selecting cytokinesis-blocked cells which can be scored for the presence of micronuclei (43, 44) were:

1. The cells should be binucleated.
2. The two nuclei in a binucleated cell should have intact nuclear membranes and be situated within the same cytoplasmic boundary.
3. The two nuclei in a binucleated cell should be approximately equal in size, staining pattern and staining efficiency.
4. The two main nuclei in a binucleated cell may touch, but ideally should not overlap each other.
5. The cytoplasmic boundary or membrane of a binucleated cell should be intact and clearly distinguishable.

Criteria for scoring micronuclei (43, 44) were:

1. Micronuclei are round or oval in shape.
2. Micronuclei are non-refractile and they can therefore be readily distinguished from artifacts.
3. Micronuclei are not linked or connected to the main nuclei.

4. Micronuclei may touch, but not overlap the main nuclei and micronuclear boundary should be distinguishable from the nuclear boundary.

5. Micronuclei usually have the same staining intensity as the main nuclei, but may have more intense staining pattern.

Intensity measurements

CHO cells were seeded on Mylar®-bottomed dishes. Forty eight hours later the cells were irradiated with 4 Gy of alpha-particles to induce micronuclei, then the cytokinesis-block method was performed, cells were fixed with methanol and stained with DAPI. Cells were visualized and digital micrographs were obtained using a Zeiss Axiophot microscope or a Zeiss inverted microscope AX-35 equipped with the appropriate filters and color digital camera. Cell nuclei and micronuclei were measured to determine the nucleus cross-sectional area versus the integral intensity of DAPI fluorescence using Scion Image® software.

TUNEL protocol

In Situ Cell Death Detection Kit (Fluorescein) by Roche Molecular Biochemicals was utilized for detection of apoptosis in cells. Adherent cells were rinsed with DPBS with calcium and magnesium and fixed with 1% paraformaldehyd solution for 40 min at room temperature. Then cells were rinsed twice with Dulbecco's Phosphate buffered saline (DPBS) and incubated in permeabilisation solution (0.1% Triton-X-100, 0.1% sodium citrate) for 5 min at 4^{o}C. After permeabilisation cells were rinsed twice with DPBS and then 100 µl of TUNEL reaction mixture and DAPI in Vectashield® was added

per sample. Later samples were visualized utilizing Zeiss Axiophot microscope or Zeiss inverted microscope AX-35 equipped with appropriate DAPI and FITC filters.

Micronucleus frequency as a function of time of incubation with cytochalasin B

CHO cells were cultured in 75 cm^2 tissue-culture flasks and passed at confluence to Mylar®-bottomed dishes. Cell density was approximately 40 cells/cm^2. Then the cells were incubated for forty eight hours to insure adequate attachment and proliferation. After that dishes were randomly divided into two groups. The first group was irradiated with 1 Gy of alpha particles, and the second group was irradiated with 4 Gy of alpha particles. Immediately after irradiation cytochalasin B was added to all dishes to the final concentration of 3 μg/ml and cells were returned to an incubator. After 3, 6, 9, 12, 16 and 24 hours of incubation with cytochalasin B, three dishes from each group were removed, fixed with 70% methanol, stained with DAPI, and then the micronuclei were scored.

Effect of radiation type on micronucleus frequency

CHO cells were cultured in 75 cm^2 plastic containers and passed at the confluence to Mylar®-bottomed dishes at approximately 50 cells/cm^2. Cells were incubated for 48 hours and then dishes were randomly divided into two groups. One group was irradiated with X rays; another group was irradiated with alpha-particles. Doses of ionizing radiation ranged from 0 to 4 Gy. Immediately after irradiation the cytokinesis-block method was performed (cells were incubated in $3x10^{-3}$ mg/ml cytochalasin B for 3 hours) and micronucleus frequency evaluated.

Persistence of micronucleus formation

CHO cells were cultured in 75 cm^2 plastic containers and passed at the confluence to Mylar®-bottomed dishes at 40 cells/cm^2 approximately. Cells were incubated for 48 hours and then randomly divided to two groups. One group was exposed to alpha particles at doses 2 and 4 Gy, another group was exposed to X rays at the same doses. Controls were sham exposed in both groups. Cytokinesis-block method was performed (cells were incubated in $3x10^{-3}$ mg/ml cytochalasin B for 3 hours) on both groups of cells in different times post irradiation: immediately after irradiation, 8 hours, 16 hours and 24 hours after irradiation. Cells were fixed with 70% methanol, stained with DAPI and micronucleus frequency was determined for each sample.

Persistence of micronucleus formation in mixed irradiated and untreated cells

CHO cells were cultured in 75 cm^2 tissue-culture vessels and passed at confluence to 16 Mylar®-bottomed dishes at approximately 6.2×10^4 cells/cm^2. Cells were incubated for 48 hours and then randomly divided to two groups: one group for alpha-particle exposure (4 Mylar® dishes) and another for X ray exposure experiment (4 Mylar® dishes). Two dishes from each group were irradiated with 4 Gy of either alpha particles or X rays, the remaining were sham exposed. Cells were incubated until they reached confluence, harvested, and then cells from two alpha-irradiated dishes and two untreated dishes were mixed in one tube and cells from four untreated Mylar® dishes were mixed in another tube. The same procedure was repeated with cells from the X ray group. Therefore, for each alpha-particle irradiated group and X ray irradiated group we had two population of cells: one of them was a mixture of irradiated and untreated cells,

the other contained only untreated cells grown under the same conditions for comparison. From each tube cells were seeded to three Mylar®-bottomed dishes at approximately 1.5×10^5 cells/cm^2 and to two plastic 25 cm^2 containers at approximately 2.3×10^3 cells/cm^2. Cells were incubated and forty eight hours later the cells that were seeded on Mylar®-bottomed dishes were removed, the cytokinesis-block procedure was performed and the frequency of micronucleated cells was determined. Cells cultured in 25 cm^2 plastic vessels were incubated for a week until they reached confluence, and then harvested. Cells from two similarly-treated containers were combined, and then cells were passed again to three Mylar®-bottomed dishes and plastic 25 cm^2 flasks approximately at the same density as before. Then all the operations were repeated to obtain second point of the curve. The whole procedure was repeated five times yielding 5-week time-course to examine micronucleus persistence in a mixed cell population (Fig. 1).

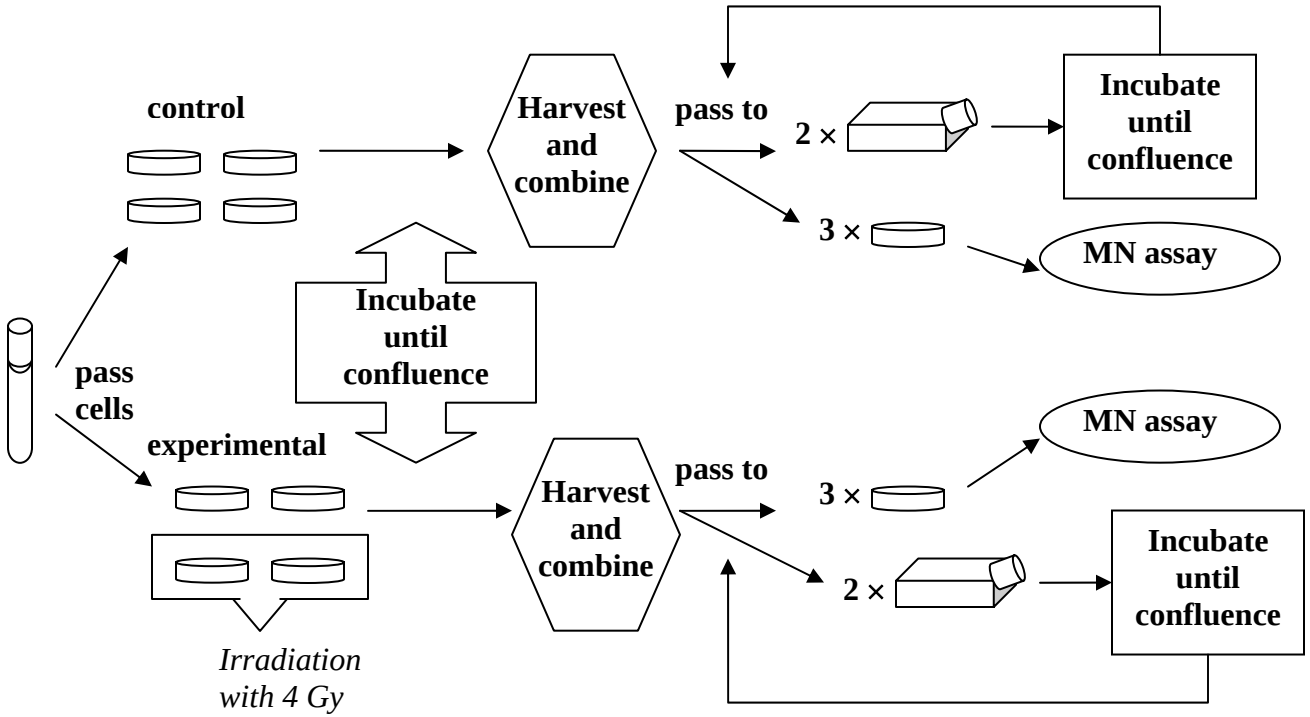

Fig. 1. Flow chart of the experimental procedure for studying the persistence of micronucleus formation in mixed irradiated and untreated cells.

Results

Many studies employ the fact that DNA content of a cells is in correlation with intensity of fluorescence staining of the nucleus of a cell in morphometric analysis (45, 46). In our study we utilized computerized analysis of DAPI-stained cells in order to find if there is a correlation between a cell size and integral intensity of DAPI fluorescence. Also we tried to establish if the integral intensity of DAPI fluorescence of the one nucleus from a normal mononucleated CHO cell is close to sum of integral intensities of the nucleus and the micronucleus in a stained binucleated cell containing micronuclei or in a stained mononucleated cell containing micronuclei. The integral intensity of fluorescence was measured in cells containing a single nucleus (Fig. 2), in cells containing one nucleus and one micronucleus (Fig. 3), and in binucleated cells with one micronucleus (Fig. 4). The results show that while the average cross-sectional area of the nucleus in the CHO cell is about (40±10) μm^2, the area of the nucleus in the cells containing one nucleus varies in a much more wide range than the area of nuclei in the binucleated cells (Figs. 2 – 4). The cross-sectional area of micronuclei varies from 2 to 12 μm^2 (Fig. 5), but there is no correlation between the area and origin of micronucleus (if the micronucleus is originated from the mononucleated or binucleated cell). The cross-sectional area of the majority of the micronuclei is 3±1 μm^2. While the integral intensity of fluorescence of micronuclei originating from single-nucleus cells increases more steeply with nuclear area than that of micronuclei originating from binucleated cells, the difference is not large. The trend of the data reveals that the fluorescent intensity of nuclei in binucleated cells increases more rapidly with area than the

fluorescent intensity of the cells containing a nucleus only (Fig. 6). Fluorescent intensity of nuclei originating from single-nucleus cells does not vary significantly with area of the nucleus.

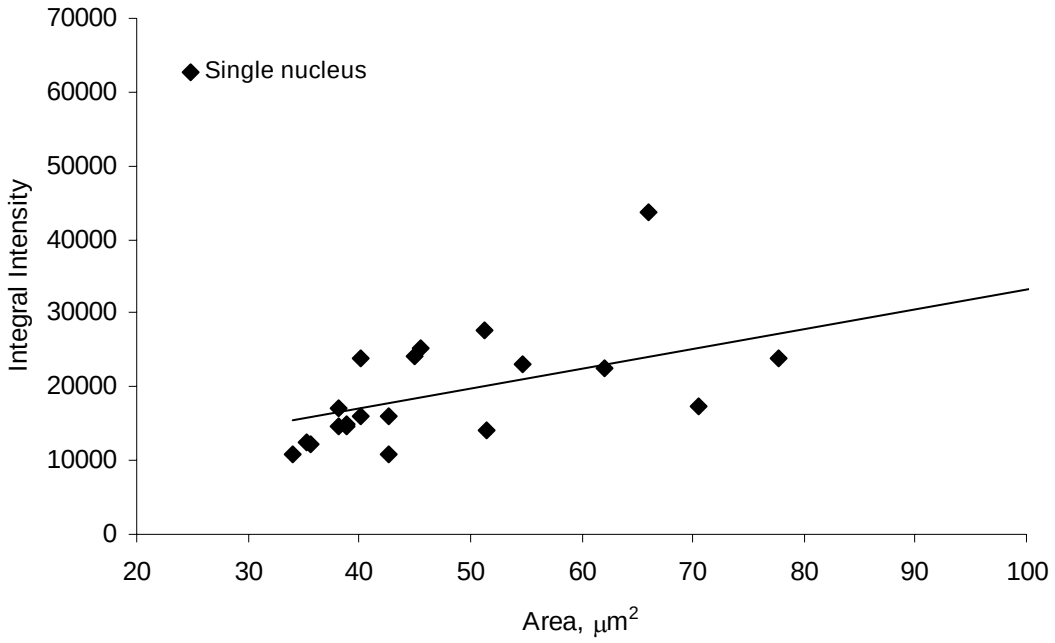

Fig. 2. Integral intensity of DAPI fluorescence in stained normal CHO cells (containing one nucleus) vs. area of the nucleus.

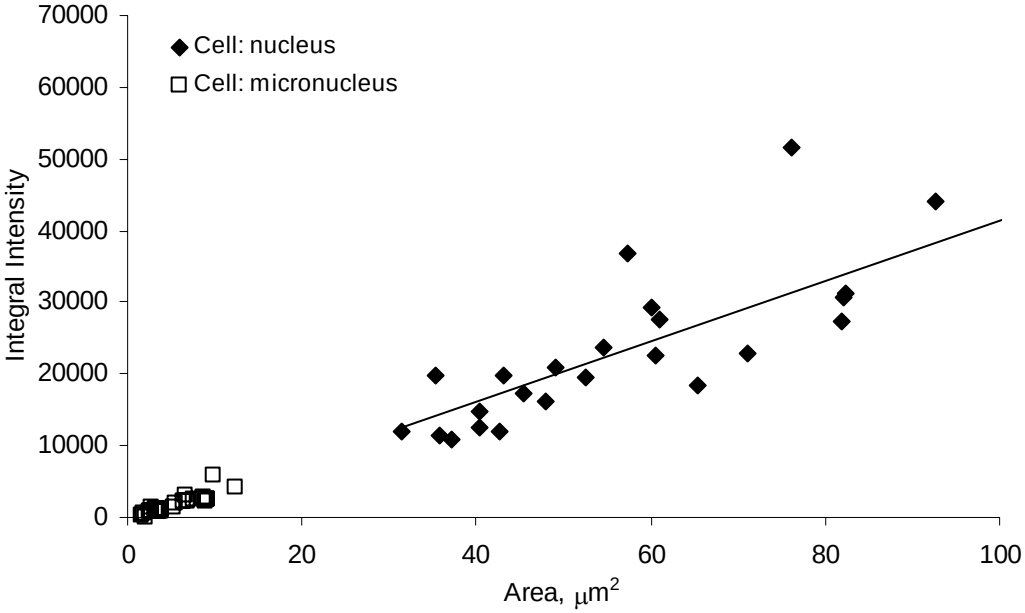

Fig. 3. Integral intensity of DAPI fluorescence in stained cells containing one nucleus and one micronucleus. Data represent intensity vs. area of nucleus (diamonds) and micronucleus (squares) in the cells.

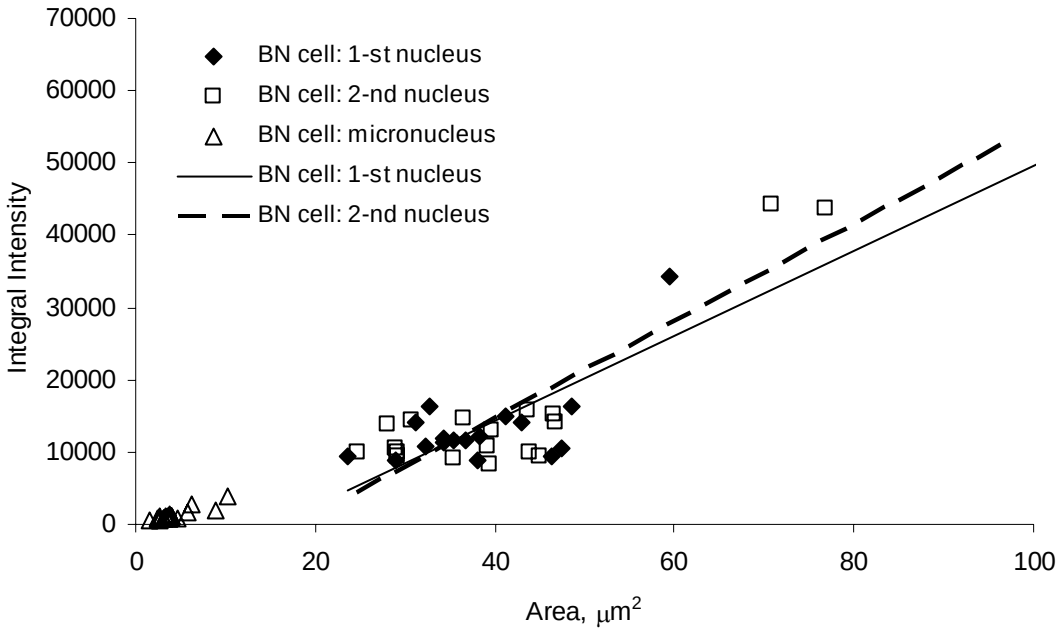

Fig. 4. Integral intensity of DAPI fluorescence in stained binucleated CHO cells containing one micronucleus. Data represent intensity vs. area of the each of two nuclei (squares and diamonds) and micronucleus (triangles) in the cells.

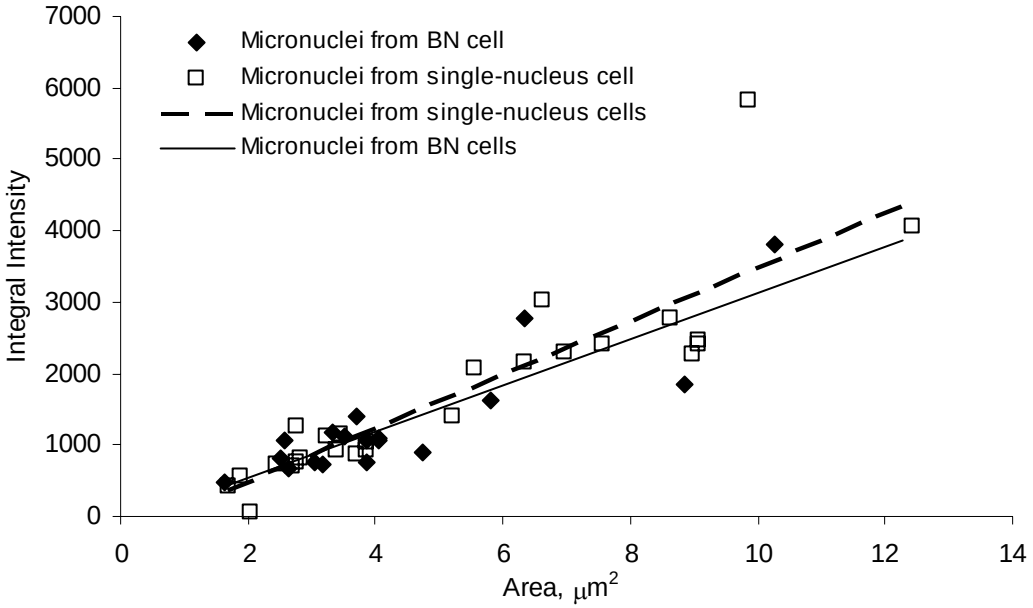

Fig. 5. Integral intensity of DAPI fluorescence of stained micronuclei originated from binucleated cell containing one micronucleus (diamonds) and from the cells containing one nucleus and one micronucleus (squares). Area represents the area of micronuclei in micrometers square.

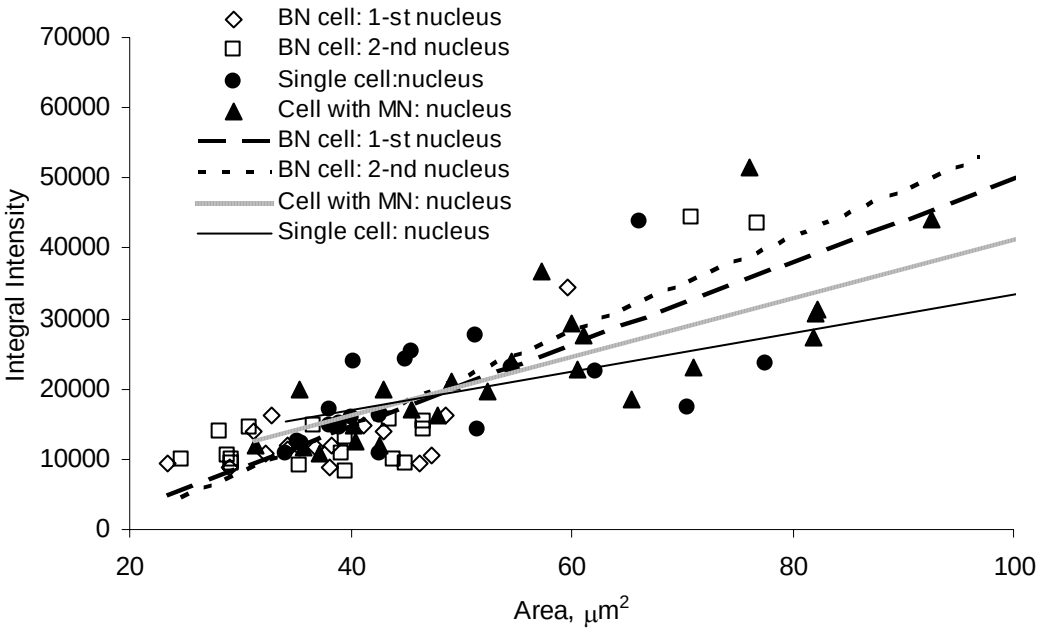

Fig. 6. Integral intensity of DAPI fluorescence in stained cells. Data represent intensity vs. area of each of two nuclei in binucleated cells (diamonds and squares), nucleus of the cells with a single nucleus (circles), and nucleus of the cells containing one nucleus and one micronucleus (triangles).

Since apoptotic bodies can be mistaken as micronuclei, the next step in our study was the assessment of the frequency of apoptosis in the cell line after irradiation. There is an extensive evidence that CHO cells, like many other cultured cells, do not undergo apoptosis (47-49), but this conclusion is based primarily on the observation that Chinese hamster ovary cells have a mutated p53 gene and that p53 is mainly responsible for apoptotic mode of death in damaged cells (50). In order to confirm this conclusion we decided to perform an assay to evaluate the number of apoptotic cells and to confirm that there are no apoptotic bodies which could be mistaken for micronuclei. Apoptosis was assayed using terminal deoxynucleotidyl transferase (TdT)-mediated biotinylated deoxyuridine triphosphate nick end-labeling (TUNEL) reaction. After visualization of the results of the assay it was confirmed that there are very few apoptotic cells in each sample (less then 1%) and there were no apoptotic bodies posing as micronuclei.

Several experiments were performed varying the times of incubation in cytochalasin B in order to assess its effect on micronucleus frequency. It was found that while the total number of binucleated cells and the total number of micronuclei increases with time of incubation in cytochalasin B, the ratio of binucleated cells with micronuclei per total number of scored binucleated cells remains more or less constant. An experiment was performed in order to confirm that the observed ratio of binucleated cells containing micronuclei to the total number of scored binucleated cells remains constant for CHO cells incubated in cytochalasin B from three to twenty four hours after radiation exposure. The result is shown in Figure 7.

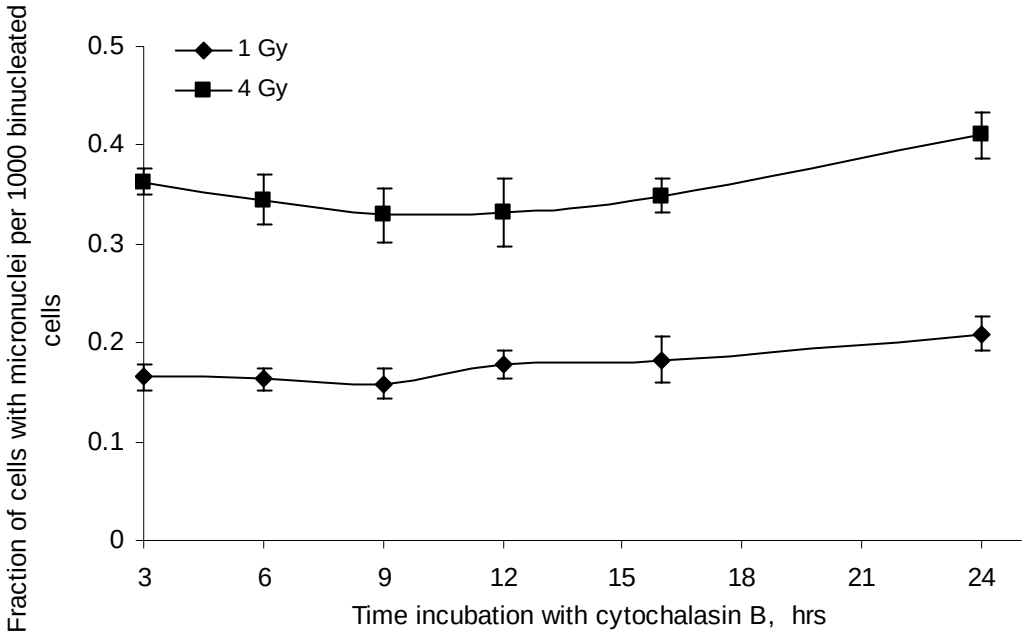

Fig. 7. Micronucleation in alpha-irradiated CHO cells depending on time of incubation with cytochalasin B. Data represent micronucleus frequency in cells irradiated with 1 Gy of alpha particles (diamonds) and in cells irradiated with 4 Gy of alpha particles (squares). Error bars represent the standard deviation of the mean from three replicate samples.

It can be seen that while the fraction of binucleated cells containing micronuclei depends on the dose of radiation; this parameter does not vary with time of incubation in cytochalasin B.

The next step in the investigation was to establish the dependence of micronucleus formation in CHO cells on the type and the dose of radiation.

The fraction of binucleated cells containing micronuclei per total number of scored binucleated cells was calculated after exposure of CHO cells to alpha particles and X rays at doses ranging from 0 to 4 Gy. As can be seen, irradiation of cells with alpha particles has a greater impact than irradiation with the same dose of X rays (Fig. 8) and the difference in micronucleus frequency between alpha-irradiated and X-ray-irradiated cells increases with dose. The micronucleus frequency of cells exposed to 1 Gy of alpha particles is 1.5 times higher than the micronucleus frequency of cells exposed to 1 Gy of X rays and almost two times higher when dose was increased to 4 Gy.

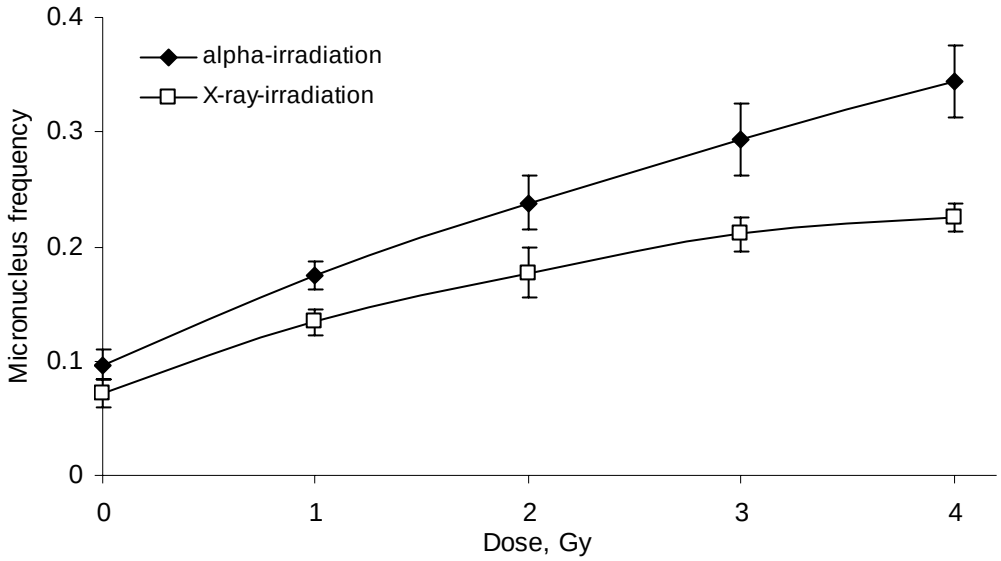

Fig. 8. Effect of radiation type on micronucleus frequency. Data represent micronucleus frequency from CHO cells irradiated on Mylar®-bottomed dishes with various doses of alpha radiation (diamonds) and X rays (squares). Error bars represent standard deviation of the mean from the three replicate samples.

In order to study the persistence of the micronucleus formation CHO cells were exposed to the range of alpha radiation or X rays from 1 to 4 Gy and micronucleus assay (cells were incubated in $3x10^{-3}$ mg/ml cytochalasin B for 3 hours) was performed immediately, 8, 16 and 24 hours after irradiation. It can be seen that the formation of micronuclei increases with time after exposure to ionizing radiation. Figure 9 depicts the time-course of the micronucleus frequency of cells exposed to 1 and 2 Gy of alpha particles or X rays. As can be seen, the micronucleus frequency increases gradually with time in both cases of exposure to X rays and alpha particles until reaching its maximum at approximately 16 hours after exposure. The overall picture is quite different in case of exposure of cells to higher doses of ionizing radiation. The micronucleus frequency of cells exposed to 3 and 4 Gy of alpha particles or X rays reaches maximum within about eight hours post-irradiation (Fig. 10). It should be noted that the kinetics of micronucleation demonstrates a clear dependence on the type of radiation also in addition to dependence on the dose of radiation. The micronucleus frequency of alpha-irradiated cells is up to 30% higher than micronucleus frequency of X-ray-irradiated cells and this trend remains the same during all 24 hours of observation.

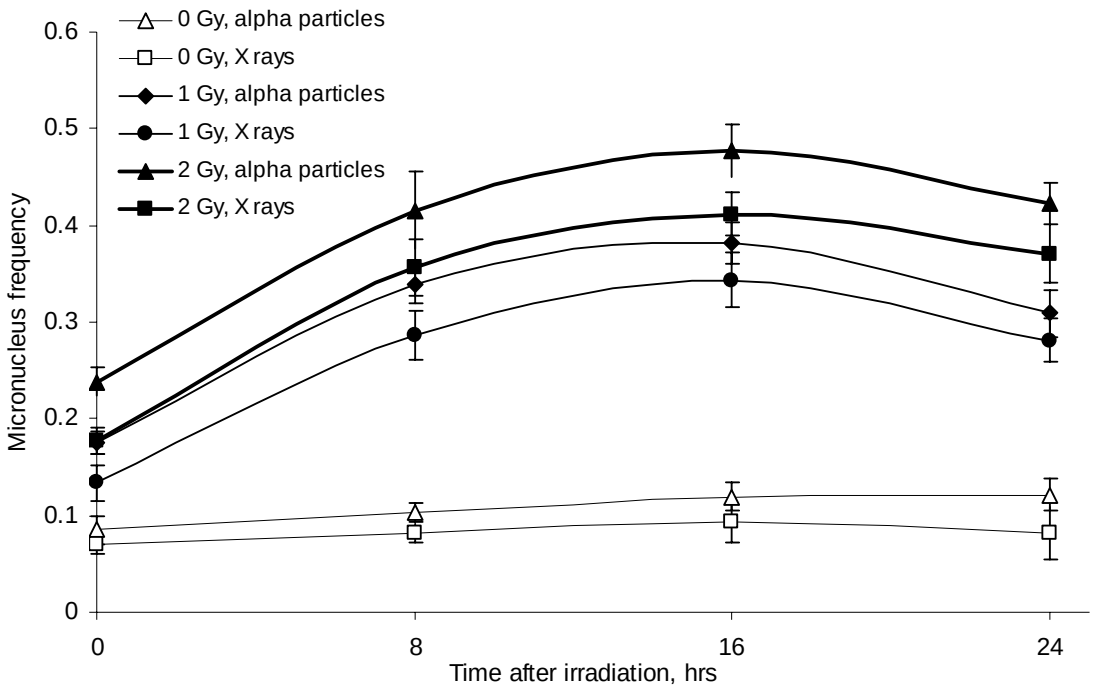

Fig. 9. Kinetics of micronucleation in low-dose irradiated cells. The data represent micronucleus frequency in cells, plated on Mylar®-bottomed dishes and exposed to 1 Gy (shaded diamonds) and 2 Gy (shaded triangles) of alpha particles or to 1 Gy (shaded circles) and 2 Gy (shaded squares) of X rays. Sham experiments were performed in alpha particle group (open triangles) and in X ray group (open squares) of dishes. Cytokinesis was blocked at different periods of time after irradiation was performed. Error bars represent the standard deviation of the mean from the three replicate samples.

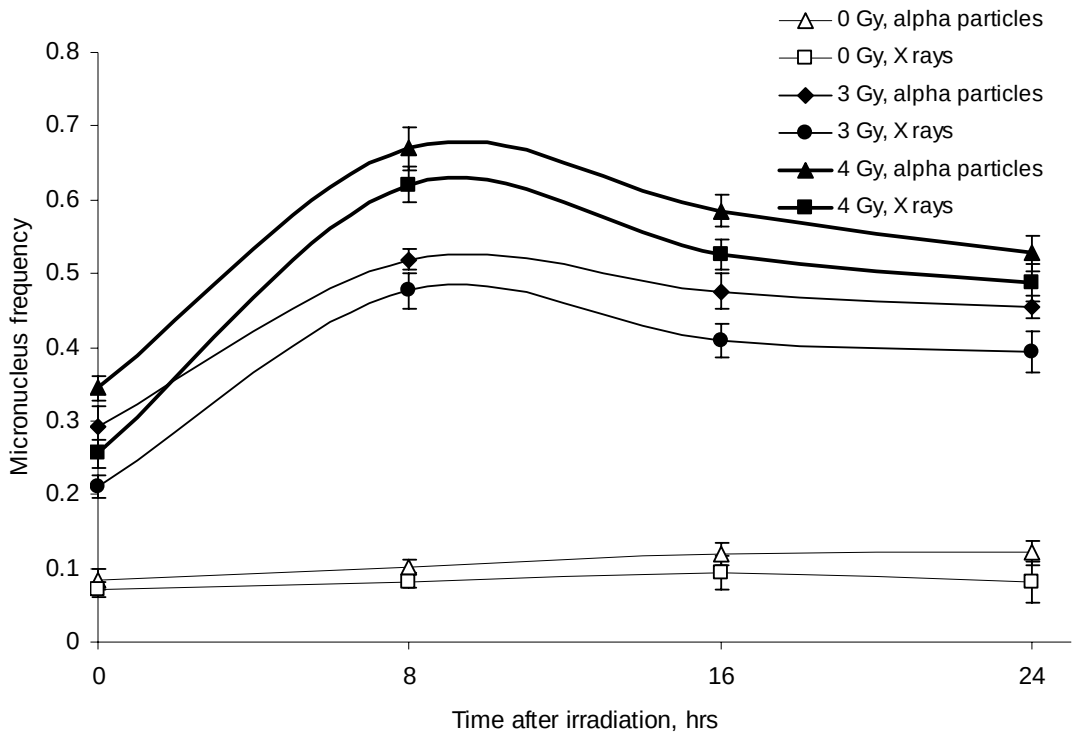

Fig. 10. Kinetics of micronucleation in high-dose irradiated cells. The data represent micronucleus frequency in cells, plated on Mylar®-bottomed dishes and exposed to 3 Gy (shaded diamonds) and 4 Gy (shaded triangles) of alpha particles or to 3 Gy (shaded circles) and 4 Gy (shaded squares) of X rays. Sham experiments were performed in alpha particle group (open triangles) and in X ray group (open squares) of dishes. Cytokinesis was blocked at different periods of time after irradiation was performed. Error bars represent the standard deviation of the mean from the three replicate samples.

To assess the possible influence of micronuclei on cells which do not originally have micronuclei a time-course study of the micronucleus frequency in a mixture of un-irradiated and irradiated cells was performed. Figure 11 represent the change in the frequency of micronucleated cells with time for mixed X-ray-irradiated CHO cells and untreated cells along with micronucleus frequency in sham-irradiated cells mixed with untreated cells. The micronucleus frequency in the irradiated mixture is significantly higher ($P_{value}<0.005$) than the micronucleus frequency of cells in mixture of sham-treated cells during first two weeks of incubation. By the third week of propagation, the micronucleus frequency stabilizes for both populations. However, the micronucleus frequency of the irradiated cell mixture remains elevated compared to the control mixture. The time-course examination of the micronucleus frequency in the alpha-irradiated mixture and in the control mixture is represented on the Figure 12. The overall picture of micronucleation pattern in a mixture of alpha-irradiated and untreated cells is very similar to that represented on the Figure 11. Figure 13 compares the micronucleus removal kinetics for both experiments.

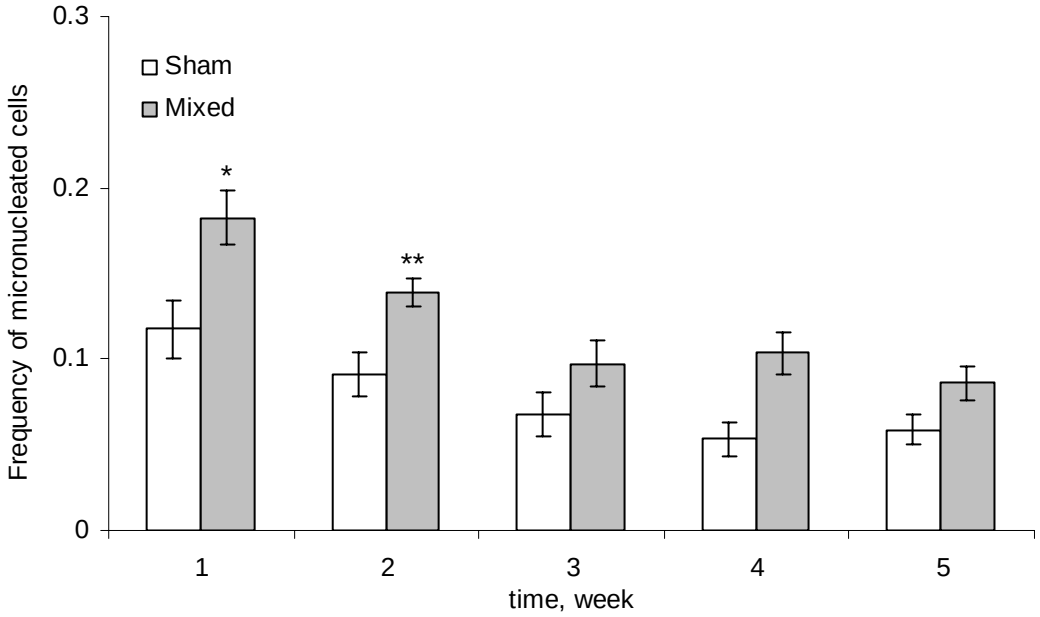

Fig. 11. Time-course of micronucleus formation in mixed untreated and low-LET irradiated cells. Cells were exposed to X rays on Mylar®-bottomed dishes and mixed with untreated cells grown under the same conditions (shaded squares). As a control, two groups of untreated cells were mixed together (open squares). Error bars represent the standard deviation of the mean from three replicate samples. Data marked (*) and (**) is significantly different from each other and from the rest of the data ($P<0.0005$).

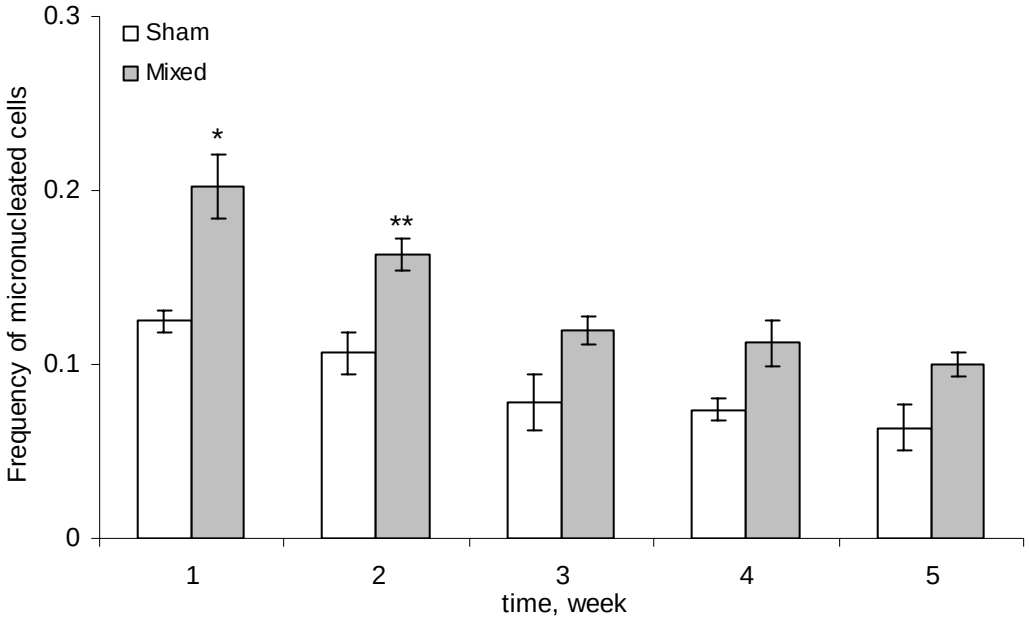

Fig. 12. Time-course of micronucleus formation in mixed untreated and high-LET irradiated cells. Cells were exposed to alpha particles on Mylar®-bottomed dishes and mixed with untreated cells grown under the same conditions (shaded squares). As a control, two groups of untreated cells were mixed together (open squares). Error bars represent the standard deviation of the mean from three replicate samples. Data marked (*) and (**) is significantly different from each other and from the rest of the data ($P<0.0005$).

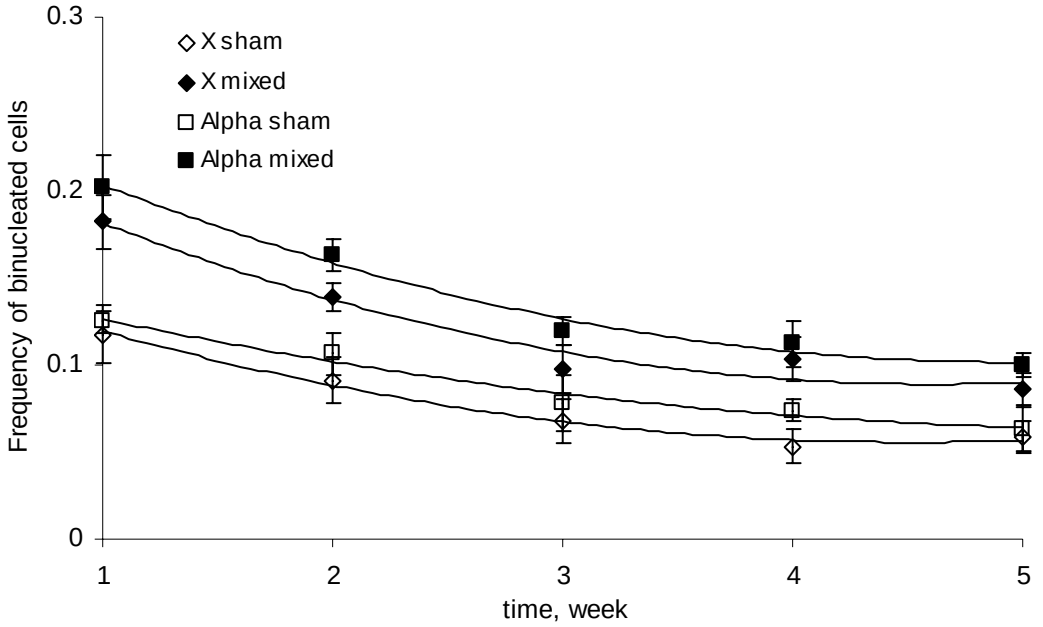

Fig. 13. Comparison of micronucleus removal kinetics in mixed CHO cells. Cells were exposed on the Mylar®-bottomed dishes to X rays (shaded diamonds) or alpha particles (shaded squares) and mixed with untreated cells grown under the same conditions. As a control two groups of untreated cells were mixed together for X-ray experiment (open diamonds) and for alpha particle experiment (open squares). Error bars represent the standard deviation of the mean from three replicate samples.

Discussion

Figure 2 depicts the distribution of the cross-sectional areas of nuclei and micronuclei in cells containing one nucleus and one micronucleus, and the relationship between an area of a nucleus and the integral intensity of fluorescence. The intensity increases linearly with increasing of the area of a nucleus or a micronucleus. It should be noted that the line representing the integral intensity of the nuclei versus area may be approximated down to zero and that parameters of micronuclei lay along the same line. At the same time the integral intensity of DAPI fluorescence of nuclei in cells containing a nucleus only (Fig. 3) does not vary much with area and the areas of nuclei are much closer than the areas of nuclei in cells containing one nucleus and one micronucleus. So we may say that the integral intensity of DAPI fluorescence represents the amount of DNA in the cell. The variations in integral intensity and areas of the nuclei in the cells containing micronuclei may be explained by random distribution of genetic material between nucleus and micronucleus in each cell resulting from the random damage of ionizing radiation. The increasing of the intensity with increasing of the area of a nucleus may also be explained by the changes in the amount of DNA in cells starting division. This speculation is supported by the fact that the integral intensity of DAPI fluorescence of nuclei in binucleated cells does not depend much on the area of the nuclei in vast majority of the inspected binucleated cells (Fig. 4) and the areas of nuclei are close (38 ± 8 μm^2). This makes sense, since cytokinesis is blocked in binucleated cells and amount of DNA in those cells did not increase. The integral intensity of DAPI fluorescence in micronuclei generally increases with the size of micronucleus

(supposedly according to amount of DNA) and does not seem to depend on the place of micronucleus origin (single-nucleus cell or binucleated cell).

In order to use the micronucleus assay more effectively and to obtain as informative results as possible we needed to choose the most effective method of collecting and representing data.

The most common method of representing results from the micronucleus assay is to count the number of micronuclei per 1000 binucleated cells. Since ionizing radiation causes a dose-dependent mitotic delay in exposed cells as the dose of ionizing radiation is increased, it becomes necessary to incubate cells with cytochalasin B for prolonged periods in order to obtain a sample with a sufficient number of binucleated cells. However, it has been reported (51, 52) that prolonged incubation with cytochalasin B leads to increased level of micronucleation and numerous chromosomal aberrations in cells. As can be seen at the Figure 7, micronucleus frequency of cells irradiated with 4 Gy of alpha particles is two times greater than the micronucleus frequency of cells irradiated with 1 Gy of alpha particles and this trend is kept more or less stable for 24 hours which is about time required for a full cell cycle. Therefore, it was established that micronucleus frequency of cells expressed as number of binucleated cells containing micronuclei per total number of scored binucleated cells can be a reliable tool for evaluation of results of cytokinesis-block test at least in CHO cells. Using this method of representation, we can select a short period of incubation of cells in cytochalasin B thus decreasing the total time required to perform a given experiment.

Figure 8 depicts the result of the irradiation of CHO cells with alpha particles (high LET radiation) or with X rays (low LET radiation). As can be seen micronucleus frequency of alpha-irradiated cells is significantly higher than micronucleus frequency of X-ray-irradiated with the same dose cells as expected from previously published results (53-55). It should be noted that the micronucleus frequency of cells exposed to alpha particles increase almost linearly with dose of ionizing radiation, while X-ray-irradiated cells demonstrate polynomial dependence. Our experiments found that exposure of CHO cells to alpha-radiation leads to 1.5- to 2-fold increase of number binucleated cells with micronuclei per total number of binucleated cells compared to exposure of cells to X rays.

Once the dose-dependence and radiation-type dependence was established for CHO cells, the next logical step was to determine how long micronuclei persist after irradiation.

Figure 9 represents the micronucleus frequency of cells exposed to relatively low doses of X rays or alpha particles. Figure 10 represent the micronucleus frequency of cells exposed to higher doses (3 and 4 Gy) of the same types of ionizing radiation.

The maximum value of micronucleus frequency is about 40% higher in case of exposure of cells to high doses of ionizing radiation compared to lower dose exposures. After 24 hours of incubation of irradiated cells the micronucleus frequency remains significantly elevated compared to the micronucleus frequency of sham exposed cells (up to 75% higher in case of high-dose of alpha radiation). Also the micronucleus frequency of cells exposed to high doses of ionizing radiation is still up to 25% higher

than micronucleus frequency of cells exposed to 1 or 2 Gy of ionizing radiation. The micronucleus frequency gradually increases after exposure of cells to lower doses of radiation and peaks at about 16 hours after irradiation (Fig. 9). At the same time the micronucleus frequency of cells exposed to acute doses of radiation increases more rapidly and peaks at about 8 hours after irradiation. Therefore, it can be concluded that low doses of ionizing radiation produce a more delayed effect compared to higher doses (Figs. 9, 10). For both cases of alpha-irradiation and X-ray-irradiation once the maximum is reached, it gradually decreases with time after exposure. The background micronucleus frequency remains more or less constant during the time of observation. The slightly higher micronucleus frequency of cells which received sham alpha-irradiation could be due to differences in handling sham samples during X-ray- and alpha-particle-irradiation (alpha-irradiation requires opened Mylar® dishes, but X-ray-irradiation allows performing of the experiment without opening dishes).

Since micronuclei are pieces of chromosomes or the whole chromosomes lost during mitosis, it might be important to follow the fate of micronuclei during subsequent cell divisions. This would reveal if there is any influence from a micronucleus on the cell where the micronucleus is situated or even on neighboring cells.

The results of the time-course study of micronucleation in mixed irradiated with X rays or alpha particles represented on the Figures 11 and 12. The overall picture is similar for both cases: initially elevated micronucleus frequency decreases slowly for the first three weeks until it reached the plateau. During the incubation time the micronucleus frequency of control cells gradually decreased and after three weeks of

incubation reached a plateau, when the micronucleus frequency was up to 40% lower than the initial micronucleus frequency (one week after the experiment began). The observed increase of the micronucleus frequency in the sham treated cells at the beginning of the experiment may be explained by the frequent use of trypsin for harvesting cells and interference with cell microenvironment. However, the level of micronucleation in mixed untreated cells remained up to 50% lower than it was in mixed untreated and irradiated cells. Figure 13 depicts comparison of micronucleus removal kinetics in mixed cells. The results show that the micronucleus frequency gradually decreases with time and reach a plateau after about 3 weeks of incubation. Kinetics in all cases is best described by polynomial equations of the second order. The micronucleus frequency in untreated CHO cells mixed with irradiated CHO cells is significantly higher (about 80%, $P_{value}<0.0005$) than the micronucleus frequency in cells in control experiment, but after 5 weeks of incubation the difference in the micronucleus frequency drops to about 35%. The normal background level of the binucleated cells containing micronuclei varies from 7% to its highest value of 10% and the micronucleus frequency five weeks after exposure of cells to ionizing radiation remains at the level of about 13%. Since the half-life of the micronuclei is around five weeks for both X rays and alpha particles experiments which is equal to about 60 cell cycles of CHO cells, we hypothesize that such a slow kinetics of micronucleus removal might indicate possible interactions between untreated and irradiated cells.

CHAPTER III

EFFECTS OF PRE-IRRADIATION OF CELL CULTURE VESSELS

Introduction

Irradiation of cells in culture as a part of many radiation biology experiments is inseparably linked to simultaneous irradiation of cell culture vessels. Research groups studying the influence of radiation on different materials report numerous changes that ionizing radiation produces in glasses and plastics (56–59). Exposure of polymer materials to high doses of radiation leads to significant changes in their chemical composition. In addition, high-dose gamma-irradiation is one of the preferred methods of tissue culture vessel sterilization. According to several studies (59, 60), exposure of polyethylene to sterilizing doses of ionizing radiation result in the formation of numerous volatile products such as benzene, quinine and many others. These radiolysis products may be detected for more then 4 weeks following irradiation of high-density polyethylene and in low-density polyethylene they are still present for more then seven months after irradiation (59). As many polymer materials, polyethylene terephtalate (Mylar®) may also undergo thermal degradation resulting in formation of acetaldehyde (61).

Very high doses of radiation cause temperature increase in the organic material samples, ionization, and excitation of molecules that can cause bond breakage in polymers. Atoms such as hydrogen, oxygen, and chlorine escape the material forming volatile molecules. At very high doses atoms escape the irradiated material, resulting in severe mass loss (57). In addition, irradiation causes changes in chemical activity and the

abundance of trace elements. Since both tissue-culture plastic and Mylar® film usually have trace levels of different chemical elements such as Pb, Cu, Fe, Sb, Ca, P and others (Manufacturer information), altered chemical activity of these elements may affect cells attached to the surface of this material. Moreover, chemical changes in irradiated medium have been reported (58). Therefore, chromosome damage in cells may be caused not only by ionizing radiation, but also by chemical agents originating from damaged culture vessels. The contribution of these factors to the cell damage may have synergetic character.

In order to study the influence of radiation on the cell environment we picked cell culture vessels that are commonly used for *in vitro* radiation experiments: Mylar®-bottomed dishes and coverslip chambers. Mylar® (62) is an organic polymer that mainly consists of carbon, hydrogen, oxygen, nitrogen and traces of some other chemical elements. Numerous studies show that radiation can cause significant changes in chemical composition of polymers (57, 58, 63, 64). Cells are in contact with the material of culture containers, and any changes in the material may influence the cells. Another common culture vessel is a glass-bottomed chamber. Borosilicate glass, which forms the bottom of this type of chamber, contains elements such as silicon, boron, sodium and aluminum.

Materials and methods

Cell culture

CHO cells were maintained in F-12 Nutrient Mixture (Ham) solution (Gibco BRL) with 10% Fetal Bovine Serum (HyClone) at 37^0C in a humidified atmosphere containing 5% CO_2. Cells were cultured in 75 cm^2 tissue-culture vessels (Costar) and passed at confluence to Mylar®-bottomed dishes or tissue-culture chambers with borosilicate glass bottoms (LabTek). The cells were seeded at a cell density of approximately 40 cells/cm^2, 48 hours prior to irradiation.

Alpha-irradiation

Curium-244 disk source (diameter 10 mm, activity 10 μCi at 3/1/98. Activity was 8.7 μCi at the time of the experiment) was used for alpha irradiation. Irradiation was carried out at room temperature with an exposure rate of 1 Gy/min. Figure 14 depicts the flow chart of the experimental procedure.

Mylar® dishes were sterilized in a dry heat oven for 12 hours at 180^0C and randomly separated into two groups. One group (Mylar® 2) of dishes was irradiated with 2 Gy of alpha particles. Immediately after irradiation unexposed cells were seeded and medium was added. Simultaneously cells were seeded on untreated dishes (designated Mylar® 1). Cells were then incubated for 48 hours at 37^0C. After incubation, cells from both treatment groups of Mylar® dishes were irradiated with alpha particles with doses ranging from 0 to 4 Gy.

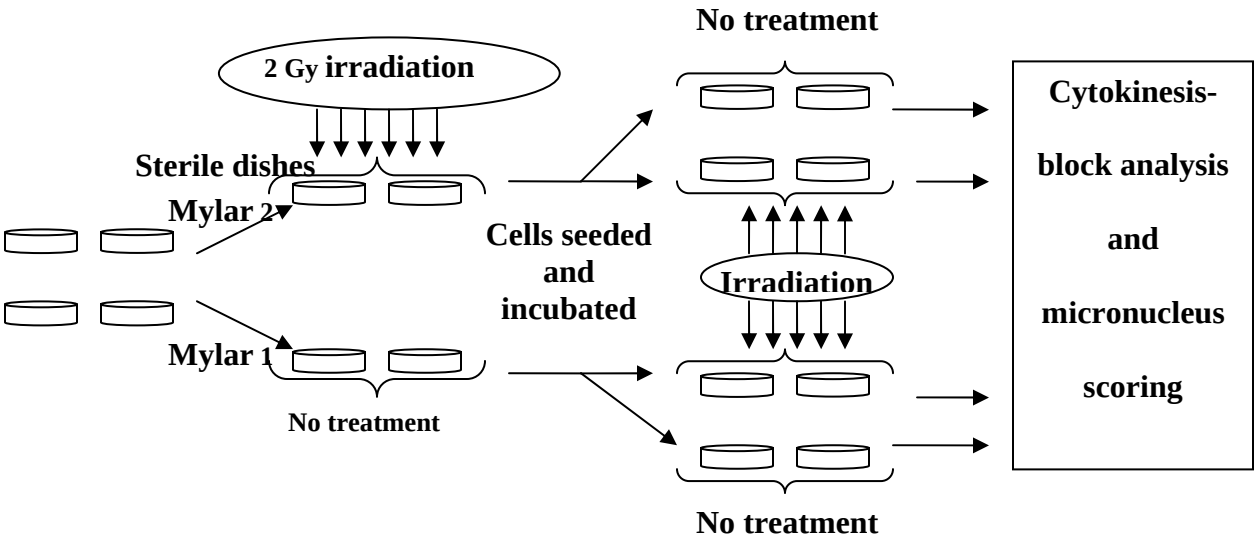

Fig. 14. Flow chart of the experimental procedure for pre-irradiation of Mylar®-bottomed dishes.

X-ray-irradiation

X-ray-irradiation was carried out using 250 kVp X rays from a Norelco X ray machine at room temperature at an exposure rate of 1 Gy/min. These sets of exposures were carried out in the same manner as the alpha-irradiation. Both Mylar® dishes and tissue-culture chambers were used as culture vessels resulting in four separate treatment groups. Setup for this experiment is identical to one for the experiment with alpha-irradiation of Mylar® (Fig.14).

One group of Mylar® dishes (*X*-Mylar®-2) and one group of tissue-culture chambers (*X*-glass-2) were irradiated with 2 Gy of X rays. Immediately after irradiation cells were seeded on both irradiated and un-irradiated (*X*-glass-1 and *X*-Mylar®-1) groups of Mylar® and tissue-culture plastic, medium was added and cells were incubated at 37^0C for 48 hours. After that cells on both types of vessel were irradiated with X-rays at doses ranging from 0 to 4 Gy. Each experiment was repeated at least two times with three replicate samples for each data point.

Time-course of persistence of micronucleus formation in cells seeded on pre-irradiated surfaces

For the time-course study of micronucleus formation on pre-irradiated surfaces three sets of culture vessels were prepared: Mylar®-bottomed dishes pre-irradiated with 2 Gy of alpha particles, Mylar®-bottomed dishes pre-irradiated with 2 Gy of X rays and glass-bottomed tissue-culture chambers pre-irradiated with 2 Gy of X rays. Cells were seeded on pre-irradiated culture dishes with different time intervals: immediately, 24, 48, 72, and 96 hours after pre-irradiation; medium was added and cells were incubated for 48 hours. Then each time group for each type of pre-irradiated vessel was randomly divided to four subgroups: the first subgroup was left untreated, the second, third and forth subgroups were irradiated with 0.5, 1, and 2 Gy respectively of the same type of radiation used for pre-irradiation of the culture vessel in this group. After that cytokinesis block method was performed and micronucleus frequency in the samples was scored (see Chapter II, pages 15-17).

Results

In order to assess the possible influence of pre-irradiated cell culture vessels on cells the micronucleus assay was performed on cells seeded on pre-irradiated with 2 Gy of alpha particles or 2 Gy of X rays Mylar®-bottom dishes and on pre-irradiated with 2 Gy of X rays coverslip tissue-culture chambers. Forty eight hours after seeding cells were irradiated with either alpha particles or X rays (according to the method of pre-irradiation of corresponding vessels) with doses ranging from 0 to 4 Gy. As a control the same procedure was performed with cells seeded on untreated surfaces.

It is well known that cells in culture tend to undergo senescence and change their characteristics and response to external agents (65, 66). Therefore, we decided to check the possible influence of the age of cells used in our experiments. Cells of two different ages (passages) were seeded onto pre-irradiated and untreated culture vessels for all treatment groups to examine any passage dependent differences. Cells of passages 5 and 15 were seeded on untreated dishes (Fig. 15) and on pre-irradiated with 2 Gy of alpha particles Mylar®-bottomed dishes (Fig. 16). After 48 hours of incubation cells were exposed to the range from 0 to 4 Gy of alpha particles and the micronucleus frequency was compared. Cells of passages 7 and 15 were seeded on untreated (Fig. 17) and pre-irradiated with 2 Gy of X rays (Fig. 18) glass-bottomed tissue culture plastic chambers. After 48 hours of incubation cells were exposed to the range from 0 to 4 Gy of X rays and the micronucleus frequency was compared. The data show no significant influence of the cell age on the micronucleus formation; therefore, the data for cells of different passages was combined.

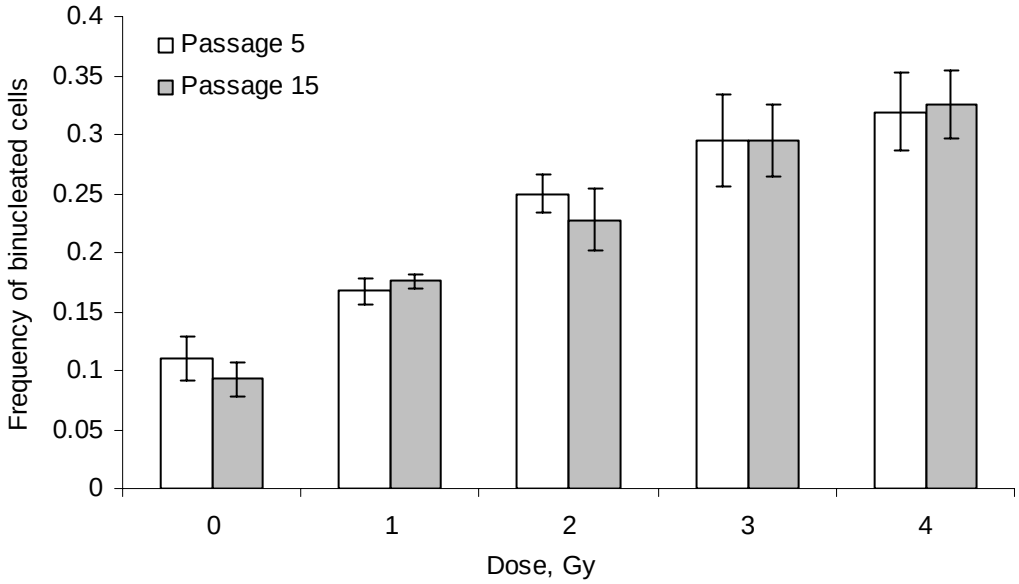

Fig. 15. Comparison of micronucleus frequency in two passages of high-LET irradiated cells. Cells of passages 5 (open bars) and 15 (shaded bars) were seeded on untreated Mylar®-bottomed dishes and after 48 hours of incubation were exposed to a range of doses of alpha radiation. Error bars represent the standard deviation of the mean from three replicate samples.

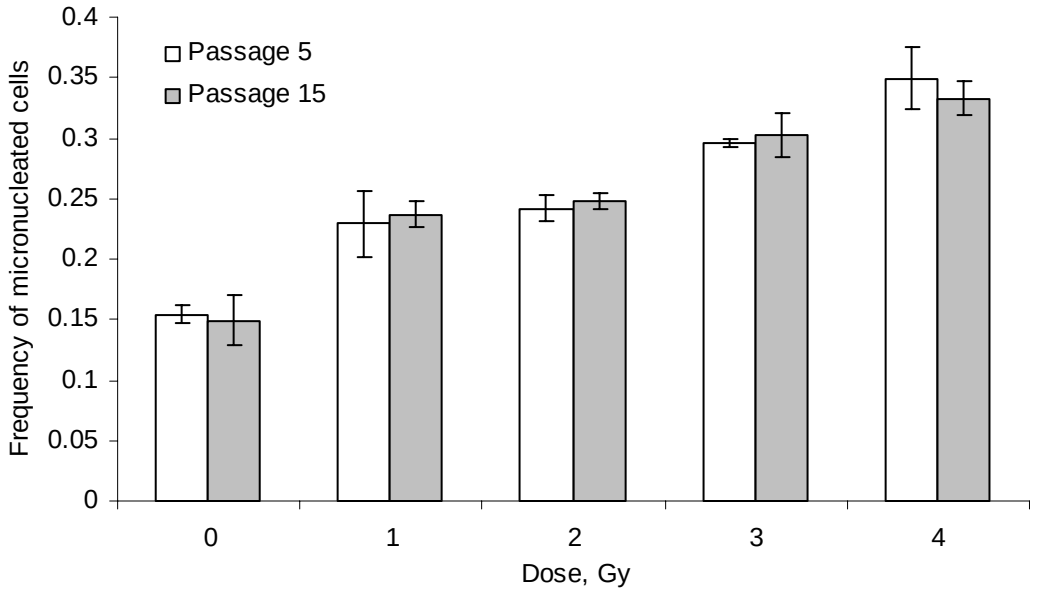

Fig. 16. Comparison of micronucleus frequency in two passages of high-LET irradiated cells seeded on pre-irradiated surfaces. Cells of passages 5 (open bars) and 15 (shaded bars) were seeded on pre-irradiated with 2 Gy of alpha particles Mylar®-bottomed dishes and after 48 hours of incubation were exposed to a range of doses of alpha radiation. Error bars represent the standard deviation of the mean from two combined separate experiments consisting from three and two replicate samples.

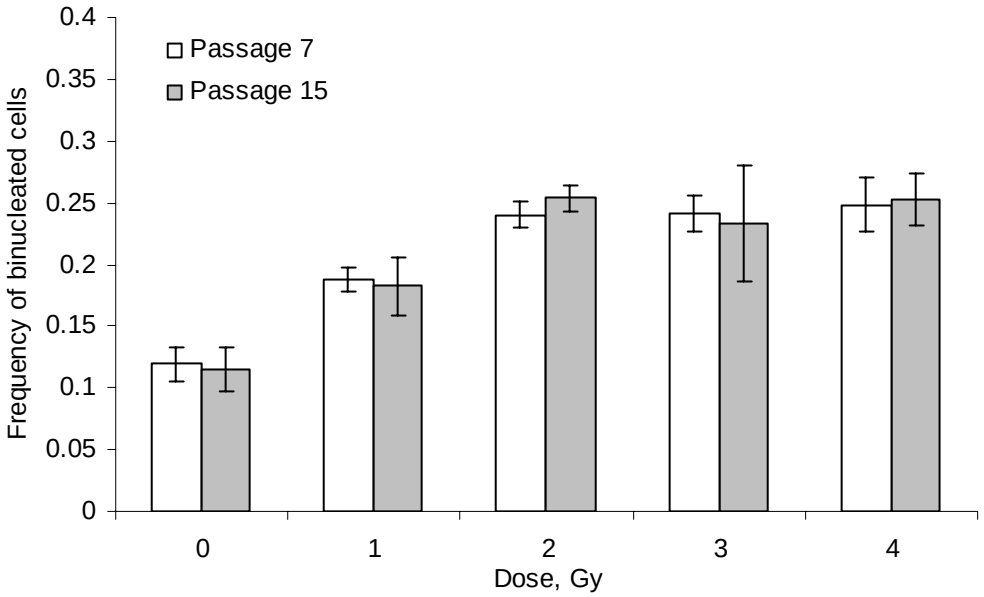

Fig. 17. Comparison of micronucleus frequency in two passages of low-LET irradiated cells. Cells of passages 7 (open bars) and 15 (shaded bars) were seeded on untreated glass-bottomed tissue culture plastic chambers and after 48 hours of incubation were exposed to a range of doses of X ray radiation. Error bars represent the standard deviation of the mean from three replicate samples.

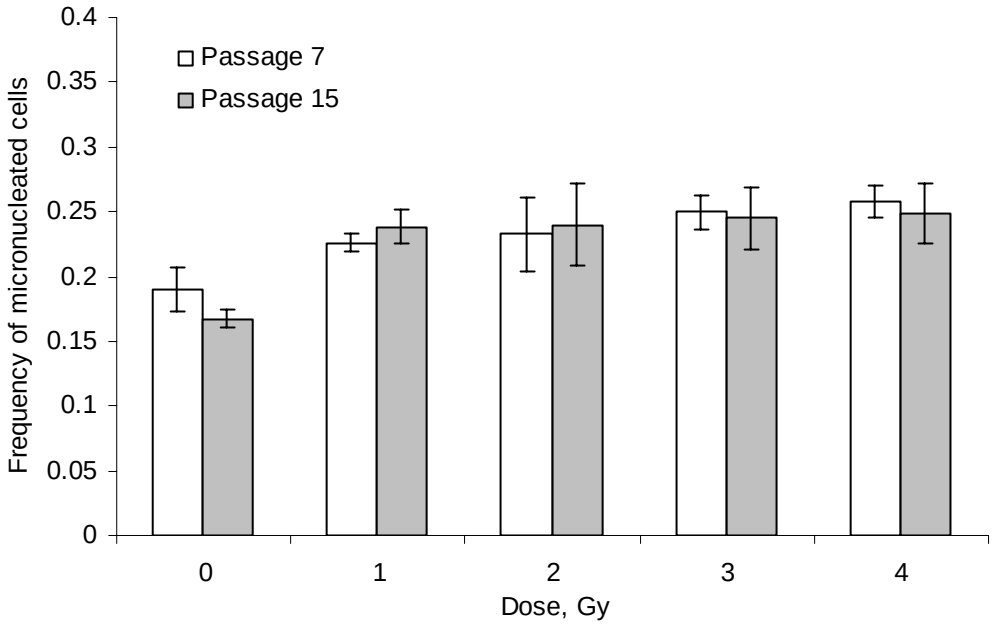

Fig. 18. Comparison of micronucleus frequency in two passages of low-LET irradiated cells seeded on pre-irradiated surfaces. Cells of passages 7 (open bars) and 15 (shaded bars) were seeded on pre-irradiated with 2 Gy of X rays glass-bottomed tissue culture plastic chambers and after 48 hours of incubation were exposed to a range of doses of X ray radiation. Error bars represent the standard deviation of the mean from three replicate samples.

Figure 19 compares the micronucleus frequency found in the alpha-Mylar® 1 and 2 groups. As can be seen, a significant increase in micronucleus frequency occurs in cells plated on pre-irradiated surfaces compared to cells plated on untreated surfaces for exposures of 1 Gy and controls (0 Gy). At these doses, the micronucleus yield is almost 1.5 times higher when cells were seeded on pre-irradiated surfaces. Generally, the micronucleus frequency of cells irradiated with alpha particles on untreated Mylar® increases linearly with dose while cells irradiated on pre-irradiated Mylar® show hypersensitivity at low doses.

In the case of X-ray-irradiation the overall picture is quite similar. Maximum increase of micronucleus frequency occurred in controls when cell-attachment surfaces were previously irradiated (Figs. 20, 21). There is a rise in micronucleus yield for the X-glass-2 group at 1 Gy (Fig. 20). The cells X-ray-irradiated on pre-irradiated vessels have a very slight change in micronucleus frequency as doses vary from 2 to 4 Gy (Figs. 20, 21). Thus, pre-irradiation of the glass is either protective for X ray doses of 3 and 4 Gy or the ability to damage a cell plateaus and additional damage is hard to differentiate. Irradiation of cells seeded on Mylar® does not cause any significant changes in micronucleus yield at doses of 1 Gy or more (Fig. 21). However, the slope of the dose response for X-ray-irradiated Mylar® is much less than that of irradiated glass-bottomed tissue-culture chambers.

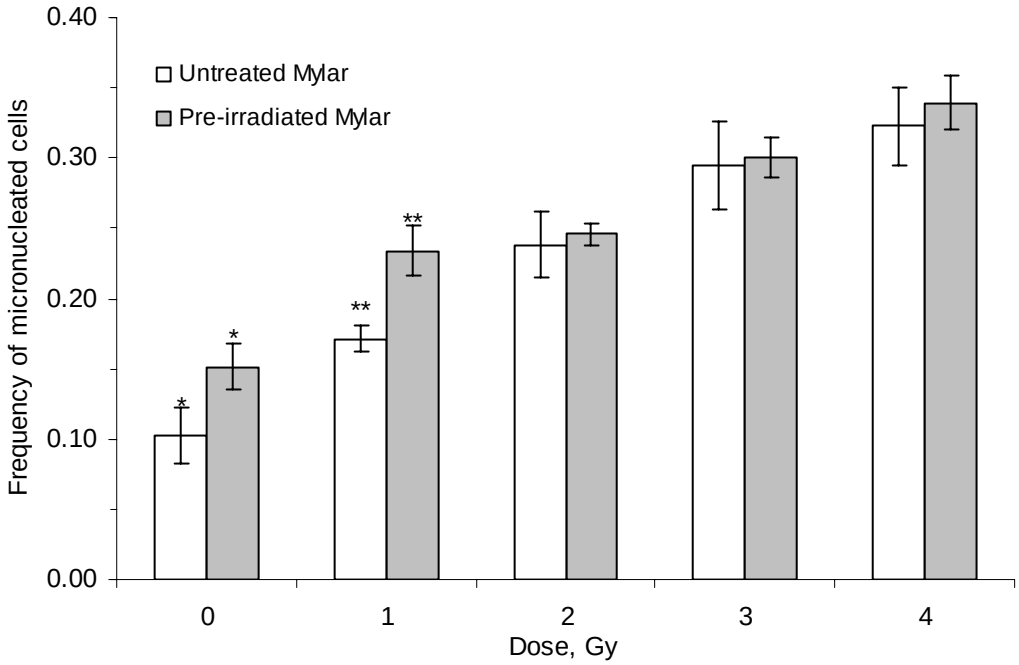

Fig. 19. Micronucleus frequency of high-LET irradiated cells on Mylar®. Combined data from passage 5 and 15 CHO cells plated on un-irradiated (open bars) or previously irradiated (shaded bars) with 2 Gy of alpha particles Mylar®-bottomed dishes. Error bars represent the standard deviation of the mean from combined experiments consisting of three replicate samples each from two separate experiments. Data marked (*) and (**) are significantly different ($P<0.005$).

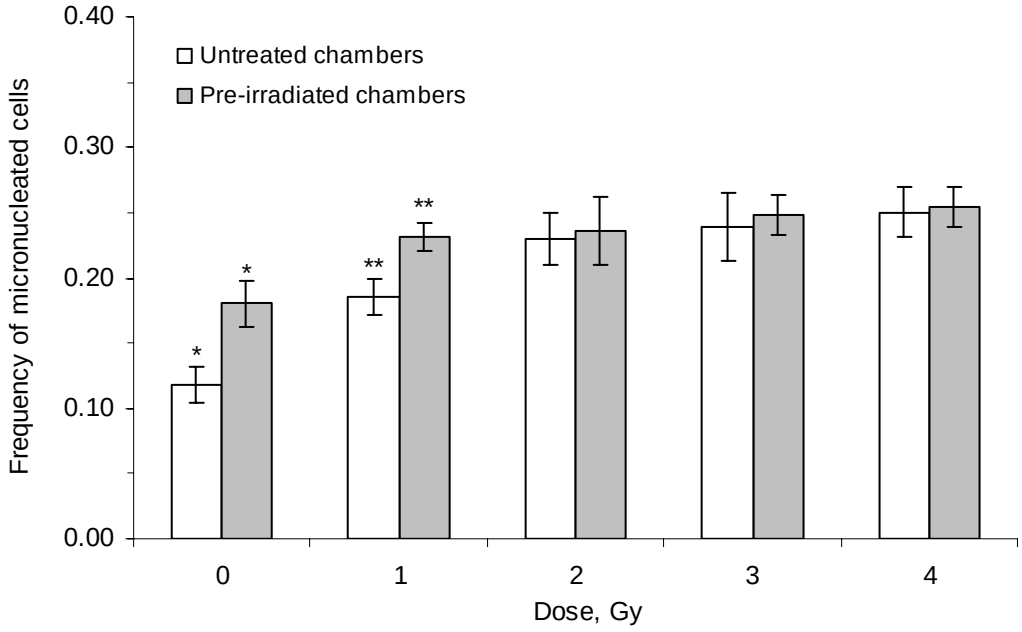

Fig. 20. Micronucleus frequency of low-LET irradiated cells on tissue culture chambers. Combined data from passage 7 and 15 CHO cells plated on un-irradiated (open bars) or previously irradiated (shaded bars) with 2 Gy of X rays glass-bottomed tissue culture plastic chambers. Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each dose point is marked with (*) and (**) ($P<0.005$) and $P=0.038$ respectively).

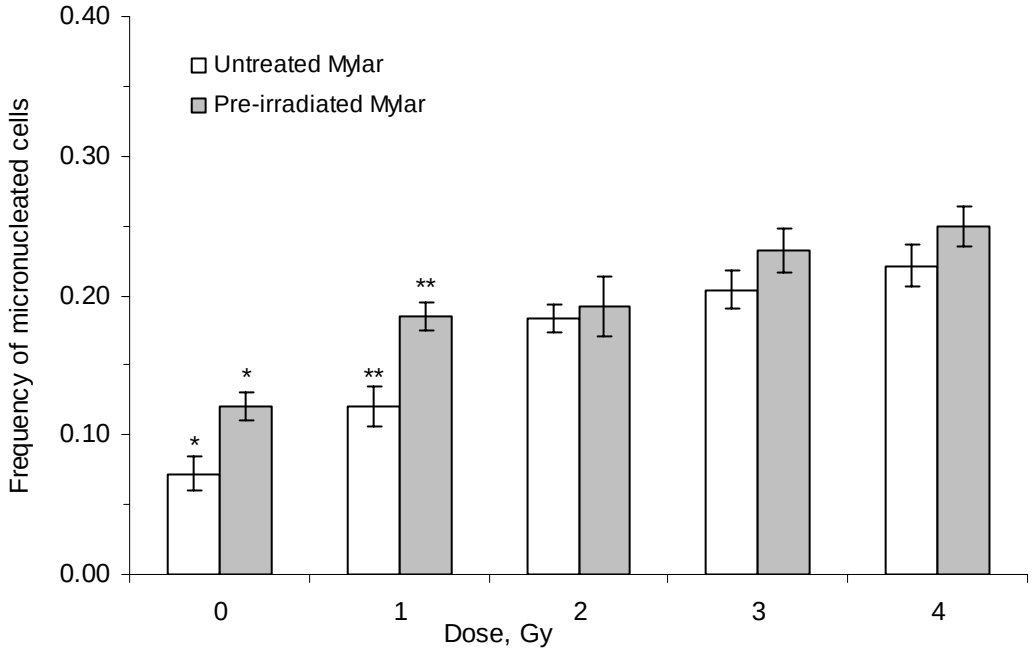

Fig. 21. Micronucleus frequency of low-LET irradiated cells on Mylar®. Passage 5 CHO cells plated on un-irradiated (open bars) or previously irradiated with 2 Gy of X rays (shaded bars) Mylar®-bottomed dishes. Error bars represent standard deviation of the mean from three replicate samples. Significantly different data for each dose point is marked with (*) and (**) ($P<0.005$).

Once again the micronucleus frequency for sham exposures on untreated Mylar® was about three-quarters of the rate observed in the α-particle experiment (Fig. 19) and the response suggests a low dose hypersensitivity on un-irradiated substrate that is eliminated (by producing micronuclei in un-irradiated cells) by pre-irradiating the substrate.

The plating efficiency on Mylar® was found to be almost 20% greater than in the tissue- culture chambers (Fig. 22). Plating efficiencies for pre-irradiated Mylar® and un-irradiated tissue-culture chambers are not significantly different. Pre-irradiated tissue-culture chambers exhibited the lowest plating efficiency.

Under the same conditions (cells seeded on the Mylar® dishes) the micronucleus frequency of alpha-irradiated cells (Fig. 19) is nearly 50% greater than X-ray-irradiated cells (Fig. 21) which is in agreement with the results obtained by other researchers for cells seeded on untreated cell vessels (52-55).

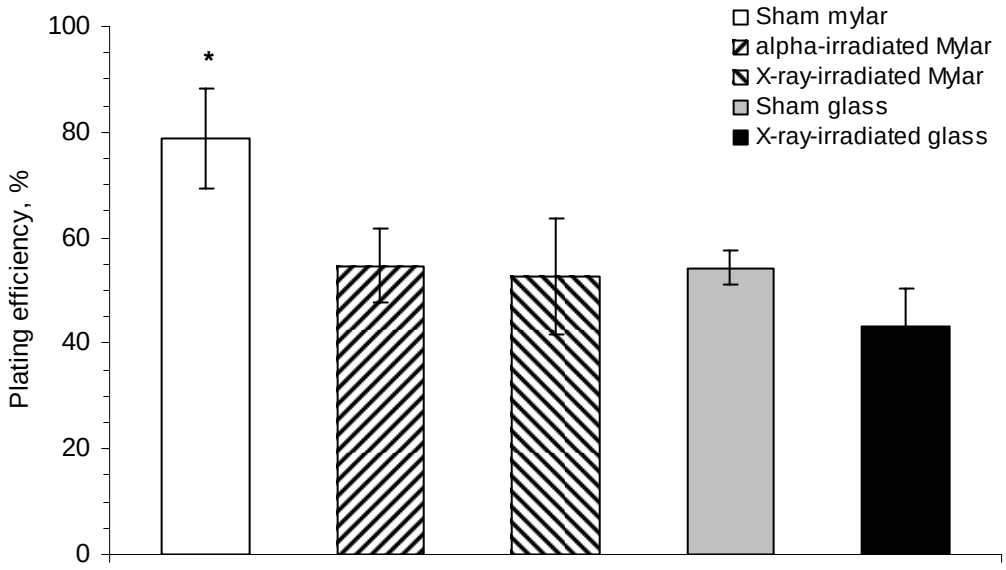

Fig. 22. Plating efficiency of cells seeded on various surfaces. Cells were seeded on un-irradiated Mylar®-bottomed dishes (open bars) or on previously irradiated with 2 Gy of X rays dexter hatching) or alpha particles (sinister hatching) Mylar®-bottomed dishes and on un-irradiated glass-bottomed tissue culture plastic chambers (cross hatching) or on previously irradiated with 2 Gy of X rays glass-bottomed tissue-culture plastic chambers (shaded bars). Error bars represent standard deviationof the mean from the three replicate samples. Data marked (*) is significantly different (P=0.025).

The next question addressed was how long the irradiated material retains the ability to influence cells seeded on its surface. Figures 23 – 25 depict the time-course of micronucleation in cells seeded on pre-irradiated surfaces at different times post-irradiation. It can be seen that in all cases cells seeded on pre-irradiated surface 24 hours after pre-irradiation show a slight increase of micronucleus frequency, but there is no significant change in the frequency of micronucleated cells during the period of observation (Figs. 23 – 25, data for un-irradiated cells). If we expose cells to alpha particles 48 hours after seeding on pre-irradiated Mylar®, we can see the dose-dependent increase in micronucleus frequency. Cells exposed to X rays on Mylar®-bottomed dishes do not show significant dose-dependent micronucleation, but in all cases micronucleus frequency of cells exposed to ionizing radiation on pre-irradiated surfaces significantly higher than micronucleus frequency of un-irradiated cells. Generally, cells seeded on all pre-irradiated surfaces and exposed to even the lowest dose develop a significant increase of micronucleus formation compared to un-irradiated cells seeded on pre-irradiated surfaces. The most interesting results are obtained from cells seeded on pre-irradiated glass-bottomed tissue-culture plastic chambers. While the micronucleus frequency of cells seeded on pre-irradiated glass after different periods of time remains more or less constant, the micronucleus frequency of cells exposed to 0.5 Gy of X rays is highest at 48 hours after pre-irradiation and the micronucleus frequency of cells exposed to 1 and 2 Gy of X rays has its maximum at 24 hours after surface pre-irradiation (Fig. 25).

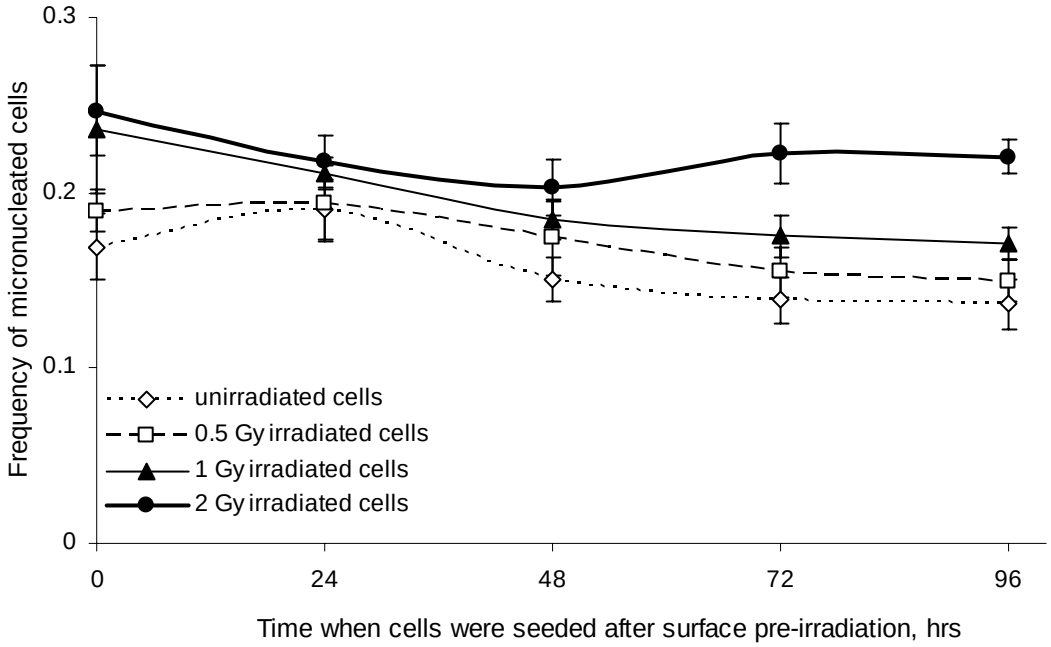

Fig. 23. Time-course of micronucleation in cells exposed to high-LET radiation on pre-irradiated Mylar®. Cells were plated on pre-irradiated with 2 Gy of alpha particles Mylar® immediately, 24 hrs, 48 hrs, 72 hrs and 96 hrs after pre-irradiation. 48 Hours after seeding cells were irradiated with 0.5 Gy (open squares), 1 Gy (shaded triangles) or 2 Gy (shaded circles) of alpha particles. Part of cells were left unirradiated for control (open diamonds). Error bars represent the standard deviation of the mean from three replicate samples each from two separate experiments.

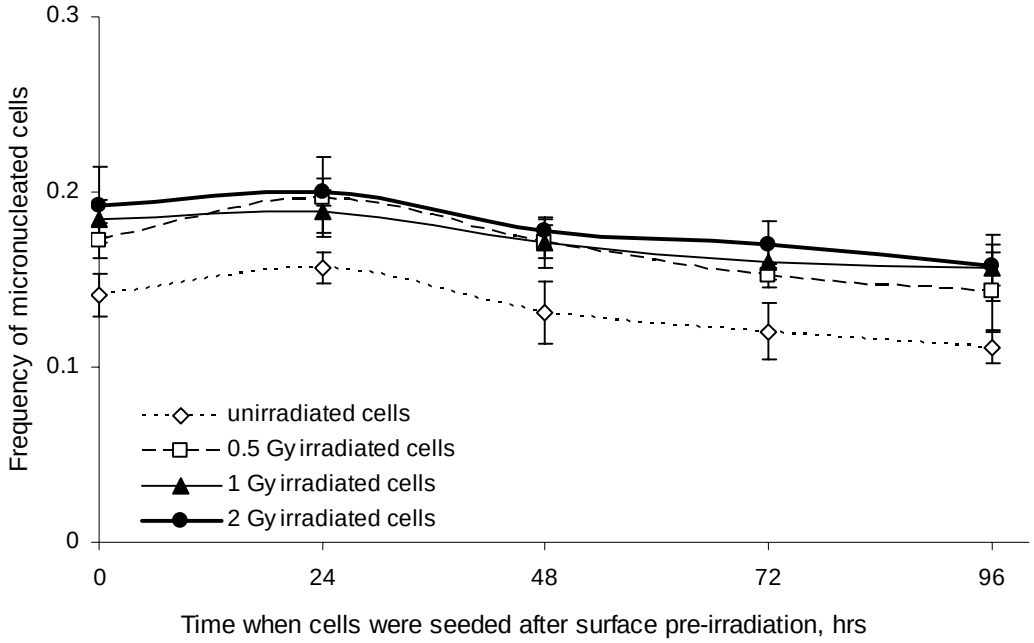

Fig. 24. Time-course of micronucleation in cells exposed to low-LET radiation on pre-irradiated Mylar®. Cells were plated on pre-irradiated with 2 Gy of X rays Mylar® immediately, 24 hrs, 48 hrs, 72 hrs and 96 hrs after pre-irradiation. 48 Hours after seeding cells were irradiated with 0.5 Gy (open squares), 1 Gy (shaded triangles) or 2 Gy (shaded circles) of X rays. Part of cells were left un-irradiated for control (open diamonds). Error bars represent the standard deviation of the mean from three replicate samples each from two separate experiments.

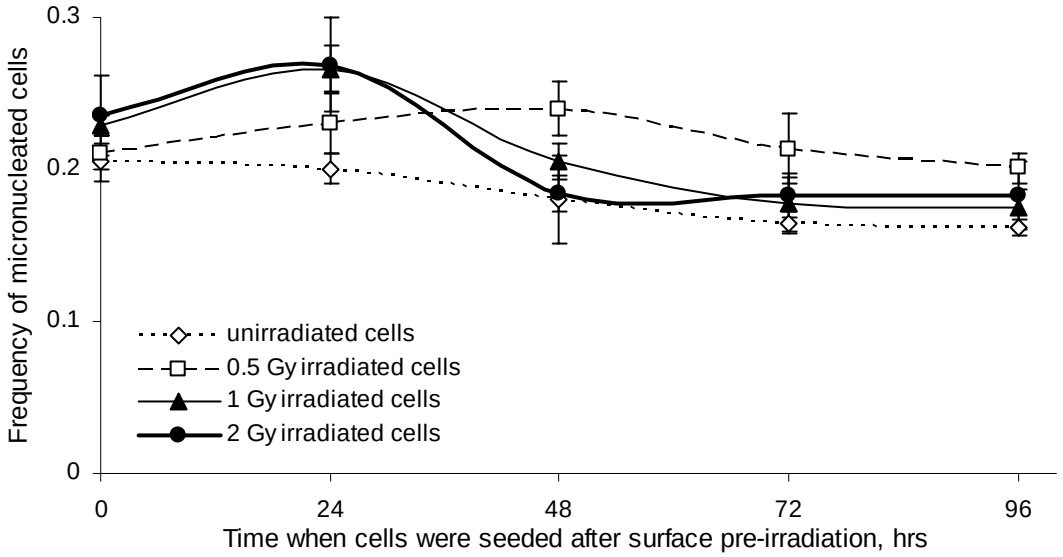

Fig. 25. Time-course of micronucleation in cells exposed to low-LET radiation on pre-irradiated coverslip tissue-culture chambers. Cells were plated on pre-irradiated with 2 Gy of X rays chambers immediately, 24 hrs, 48 hrs, 72 hrs and 96 hrs after pre-irradiation. 48 Hours after seeding cells were irradiated with 0.5 Gy (open squares), 1 Gy (shaded triangles) or 2 Gy (shaded circles) of X rays. Part of cells were left unirradiated for control (open diamonds). Error bars represent the standard deviation of the mean from three replicate samples each from two separate experiments.

Discussion

Irradiation of cell containers is an inevitable part of any radiation experiments which are conducted *in vitro.* In some materials the changes produced by a single particle track are sufficient to increase the susceptibility of the material to chemical etching. Such changes may also influence the number and quality of cell attachment sites available on a surface. And of course chemical changes in high dose irradiated medium have also been reported (58). Cells are in contact with the material of culture containers and nutrient medium, and any changes in this environment may influence the response of the cells. Therefore, cells irradiated on an artificial substrate must contend with a number of insults resulting from environmental changes induced by irradiation and which may result in chromosome damage. The reaction to the same radiation treatment may be different, depending on the type of material utilized for cell culturing, method of the cell culture vessel sterilization, and the conditions of the experiment.

The results of the present experiment demonstrate that at doses below 2 Gy, the irradiation of cell-culture vessels has a significant impact on micronucleation in CHO cells. Mylar® dishes and tissue-culture chambers were irradiated prior to plating of cells on their surfaces and micronucleus yield was compared to the data obtained when cells were seeded on the untreated surfaces. While micronucleus frequency of cells plated on untreated surfaces is in agreement with existing data (12, 33, 68), micronucleus yield of cells plated on the pre-irradiated surfaces is higher. The most significant difference in micronucleus frequency occurs in the region of doses from 0 to 1 Gy. The micronucleus frequency of cells seeded on the untreated surfaces is gradually increasing with dose

while micronucleus frequency of cells seeded on the pre-irradiated surfaces shoes no significant changes when dose increases from 1 Gy to 4 Gy. It may be suggested that the pre-irradiation of cell-culture vessel surfaces has greater impact on micronucleus induction than the irradiation of the cells at doses of less than 1 Gy. It is likely that changes in the chemical stability of elements composing Mylar® and glass may alter the chemical balance of cells plated on their surfaces causing chromosome breakage and loss. It should be noted that X-ray-irradiation of cells seeded on Mylar® dishes induces much lower micronucleus yield than alpha-irradiation of cells also plated on Mylar® (Figs. 19, 21), data for un-irradiated Mylar®). Such a trend was expected since the biological effectiveness of alpha-particles is higher than that of X rays for most endpoints in eukaryotic cells. At the same time the micronucleus frequency of cells X-ray-irradiated in tissue-culture chambers is close to the micronucleus frequency of alpha-irradiated cells on Mylar® dishes (Figs. 19, 20; data for un-irradiated surfaces). This supports the idea that there are several mechanisms responsible for radiation cell damage: the first one is based on direct impact of radiation, the second is based on indirect radiation damage from free radicals and the third is an indirect mechanism based on the chemical interactions with material of the cell culture vessels.

The results of these experiments also indicate that the baseline micronucleus frequency in cells seeded on Mylar® (Figs. 19, 21, data for 0 Gy) is comparable to the micronucleus frequency of cells seeded in tissue-culture chambers (Fig. 20, data for 0 Gy). The plating efficiency of cells seeded in untreated tissue-culture chambers is about the same as the plating efficiency of cells seeded on irradiated Mylar® (Fig. 6).

Therefore we may conclude that glass-bottomed tissue culture chambers are less acceptable for informative radiation experiment than Mylar®-bottomed dishes. The difference is probably because of the properties of glass bottom which affect attachment of CHO cells and normal colony formation.

When the persistence of elevated micronucleus frequency in cells seeded on pre-irradiated surfaces at different time after pre-irradiation (Figs. 23 – 25) was compared, it can be seen that the micronucleus frequency of cells depends not only from the type of ionizing radiation, but also from the type of culture vessel. The micronucleus frequency of cells irradiated on pre-irradiated Mylar®-bottomed dishes does not show significant dependence on the dose of radiation. This leads us to the conclusion that the main damage to the cells irradiated on the artificial surfaces is not due to radiation. The micronucleation pattern of cells irradiated on pre-irradiated glass-bottomed tissue culture plastic chambers support this hypothesis. Our previous experiments show that cells seeded on glass-bottomed chambers show unusually high micronucleus frequency after exposure to X rays. Figure 25 depicts the difference in time of maximum level of micronucleation in cells irradiated on pre-irradiated glass-bottomed plastic. It can be seen that even a low dose of radiation such as 0.5 Gy leads to increased micronucleus frequency in cells seeded on pre-irradiated surface after 48 hours after pre-irradiation, while the cells exposed to a higher dose of X rays (1 Gy and 2 Gy) result in increased micronucleus formation in cells seeded 24 hours after surface pre-irradiation.

As a result of the foregoing experiments we may conclude that cells seeded on pre-irradiated Mylar® demonstrate significantly elevated micronucleus frequency

compared to cells seeded on untreated surfaces (for alpha-irradiation experiment P_{value}=0.04, for X-ray-irradiation on Mylar® P_{value}=0.01). Based on statistics we cannot say that micronucleus frequency of cells seeded on pre-irradiated glass-bottomed tissue culture plastic chambers is significantly higher than micronucleus frequency of cells seeded on untreated chambers for all doses. However, un-irradiated cells and cells irradiated by a low dose of X rays (1 Gy) on pre-irradiated glass-bottomed chambers demonstrate significantly elevated micronucleus frequency compared to un-irradiated and irradiated with 1 Gy of X rays cells seeded on untreated surfaces (P_{value}<0.005 in both cases). Pre-irradiated surfaces retain the ability to induce elevated micronucleus frequency for at least a week after pre-irradiation (Figs. 23 – 25).

The results of the experiments show that the data obtained using the micronucleus assay should be interpreted with caution. We found that CHO cells are sensitive to the effects of radiation on their surroundings. Therefore, it might be difficult to establish whether the reason for observed cell damage is due to direct cell irradiation or from some reaction from radiation-damaged cell containers or as a result of bystander effects.

CHAPTER IV

EFFECTS OF IRRADIATED MEDIUM

Introduction

Ionizing radiation causes DNA damage in two general ways. The first one is the direct interaction with DNA and the second one is indirect damage caused by free radicals resulting from the radiolytic dissociation of water surrounding DNA (68). The main radiolysis products are hydroxyl radicals, hydrogen atoms and hydrated electrons which are chemically active themselves and may produce other highly reactive species such as peroxide and superoxide (69). All these compounds have strong oxidative ability and react with surrounding molecules resulting in variety of biochemical reactions (70, 71). Since biological materials are 70-90% water, the indirect effects of ionizing radiation are very important in radiation biology.

Many radiation experiments in culture involve simultaneous irradiation of nutrient medium and cells. The assessment of interactions of cells with irradiated medium could be very important, since numerous changes in water-based solutions exposed to ionizing radiation have been reported (72-74). It has been shown that exposure of cultured cells to irradiated medium induces mitotic delay, affects their growth and reproductive characteristics (73, 75). Therefore, we decided to investigate the influence of irradiated medium on CHO cells utilizing micronucleus assay.

Materials and methods

Cell culture

Chinese Hamster Ovary (CHO) cells from American Type Culture Collection were maintained in F-12 Nutrient Mixture (Ham) solution (Gibco BRL) with 10% Fetal Bovine Serum (HyClone) at 37 ^{0}C in a humidified atmosphere containing 5% CO_2. Cells were cultured in 75 cm^2 plastic containers (Costar) and passed at confluence to Mylar®-bottomed dishes or plastic tissue-culture chambers with borosilicate glass bottoms (LabTek). The cells were seeded approximately 40 cells/cm^2 forty eight hours prior to irradiation.

Flow cytometry experiment

For the cell-cycle distribution study cells were seeded on untreated or pre-irradiated with 2 Gy of alpha particles Mylar®-bottomed dishes and forty eight hours later were irradiated with 1 Gy or 4 Gy of alpha radiation. Sham irradiation of samples was performed in both groups of cells seeded on re-irradiated and untreated Mylar®. Cells were harvested at different time after exposure: immediately, 8 hours, 16 hours and 24 hours later, and diluted to prepare a suspension $1x10^7$ cells/ml. Harvested cells were fixed with 70% ethanol while shaking vigorously, and then cells were incubated at 4^{o}C for several days. On the day of the analysis, cells were centrifuged and resuspended in 400 µl DPBS, then 50 µl of propidium iodide stock solution and 50 µl RNAase A stock solution (1 mg/ml) were added and cells were incubated for 30 min at 37^{o}C.

Cells were analyzed on a FACSCalibur (Becton Dickinson Immunocytometry Systems, San Jose, CA) flow cytometer, equipped with a 15 mW air-cooled argon laser,

using CellQuest (Becton Dickinson Immunocytometry Systems) acquisition software. Propidium iodide fluorescence was collected through a 585/42-nm bandpass filter, and list mode data were acquired on a minimum of 5,000 single cells defined by a dot plot of PI-width versus PI-area. Data analysis was performed in ModFit LT (Verity Software House, Topsham, ME) using the auto-detection feature to model debris and cell aggregates.

Alpha-irradiation

Curium-244 disk source (diameter 10 mm, activity 10 μCi at 3/1/98. Activity was 8.7 μCi at the tome of the experiment) was used for alpha-irradiation. Irradiation was carried out under room temperature at an exposure rate of 1 Gy/min. Fig. 23 represents the setup for the experiment. Mylar® dishes were sterilized in a dry heat oven for 12 hours at 180^0C and randomly separated into two groups. One group of dishes was filled with warmed to 37^0C nutrient medium and irradiated with 2 Gy of alpha particles. Immediately after irradiation dishes were randomly divided to two groups. Unexposed cells were seeded on one group of the medium-filled Mylar® dishes ("Mylar®-2" group). Pre-irradiated medium was carefully removed from the second group of Mylar® vessels and transferred into fresh sterile Mylar® dishes ("Transferred medium" group), and then cells were seeded. Simultaneously cells were seeded on untreated dishes filled with medium under the same conditions ("Mylar® 1" group). Cells were then incubated for 48 hours at 37^0C. After incubation, cells on all groups of Mylar® dishes were irradiated with alpha-particles with doses ranging from 1 to 4 Gy. Some dishes (controls) with plated cells were left un-irradiated in all groups.

X-ray-irradiation

X-ray-irradiation was carried out using 250 kVp X rays from a Norelco X ray machine at room temperature at an exposure rate of 1 Gy/min. These sets of exposures were carried out in the same manner as the alpha-irradiation. Both Mylar® dishes and tissue-culture chambers were used as culture vessels resulting in six separate treatment groups. Setup for this experiment is identical to one for the experiment with alpha-irradiation of Mylar® (Fig. 26).

One group of Mylar® dishes (*X*-Mylar®-2) and one group of tissue-culture chambers (*X*-glass-2) were filled with warm nutrient medium and irradiated with 2 Gy of X rays. As for the alpha-irradiation experiment, pre-irradiated medium was transferred from part of the glass-bottomed tissue-culture chambers and Mylar®-bottomed dishes to the fresh vessels of the same type ("Transferred medium" groups). Immediately after that cells were seeded on all (*X*-glass-1, *X*-Mylar®-1, and Transferred medium) groups of Mylar® and tissue-culture plastic and cells were incubated at 37^0C for 48 hours. After that cells on both types of vessel were irradiated with X-rays at doses ranging from 0 to 4 Gy. Each experiment was repeated at least two times with three replicate samples for each data point.

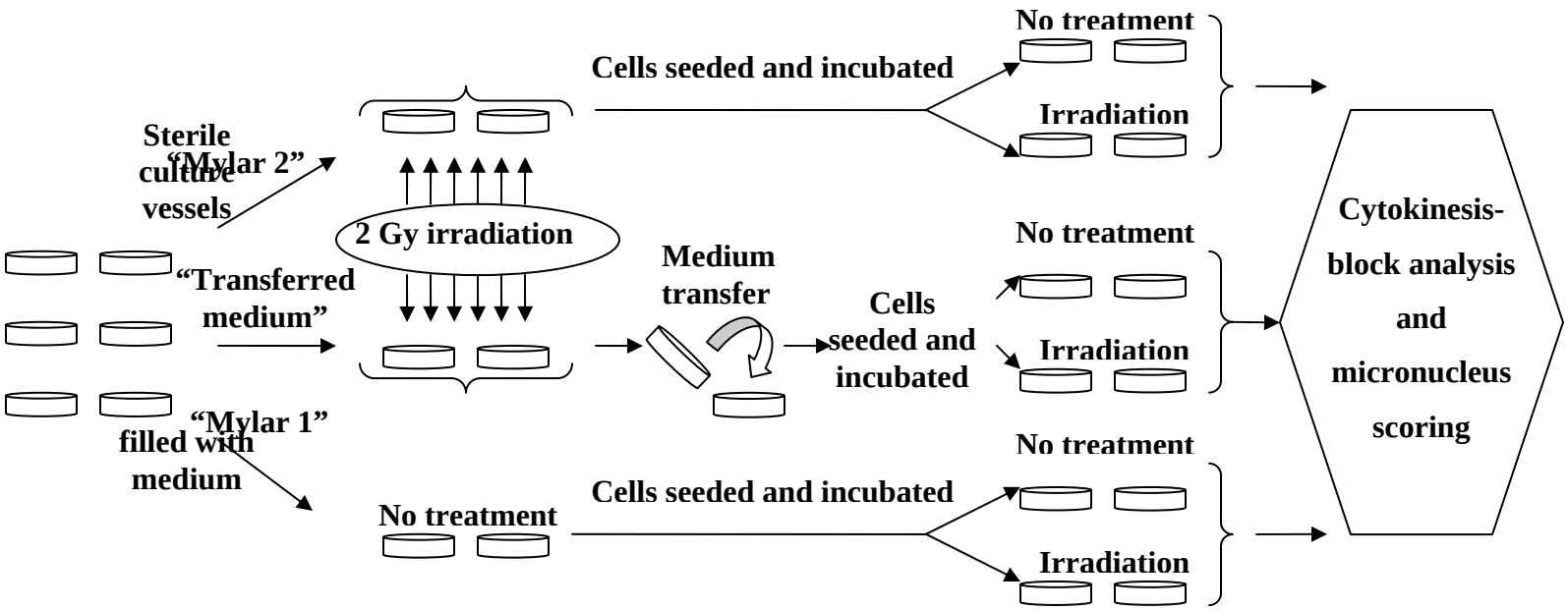

Fig. 26. Flow chart of the experimental procedure for pre-irradiation of Mylar®-bottomed dishes filled with nutrient medium.

Results

The exposure of cells to ionizing radiation induces mitotic delay arresting cells in the current phase of the cell-cycle in order to perform repair of cell damage due to radiation. Our concern was that the pre-irradiation of cell substrate might induce a significant cell-cycle delay, which could alter the cell response and lead us to invalid conclusions based on observed micronucleus frequency. We decided to check the possible influence of pre-irradiation of cell substrate on the cycle of cells seeded on pre-irradiated surfaces.

The results show that cells seeded in irradiated medium and incubated for forty eight hours and then exposed to different doses of ionizing radiation demonstrate significant mitotic delay (Fig. 27). Un-irradiated cells seeded in irradiated medium exhibited about 25% of binucleated cells after two days of incubation. The percent of binucleated cells was two times lower (about 12%) when cells were exposed to 1 or 2 Gy of alpha particles after 48 hours of incubation and about 3 times lower (about 7%) when cells were exposed to 3 or 4 Gy of alpha radiation. However, the results were not significantly different from the results obtained for mitotic delay in cells irradiated in untreated nutrient medium.

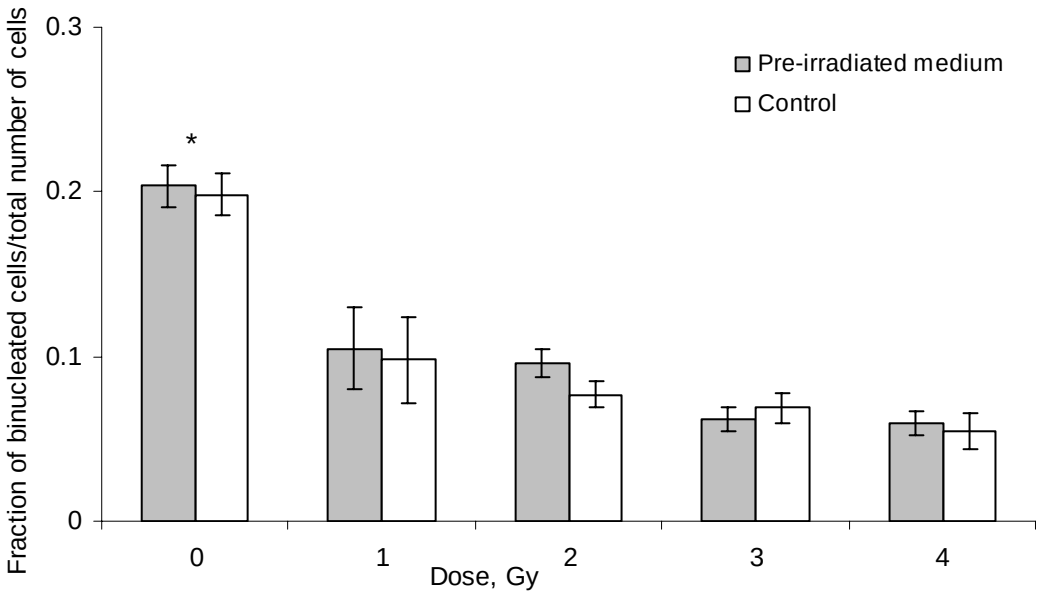

Fig. 27. Mitotic delay in irradiated CHO cells. Cells were seeded in pre-irradiated with 2 Gy of alpha-particles nutrient medium (shaded bars) or in untreated medium (open bars) and 48 hours later exposed to different doses of alpha radiation. Data labelled with (*) is significantly different from other data P_{value} is < 0.0005.

The flow cytometry of cells seeded on pre-irradiated surfaces (Fig. 28) show that there is a cell-cycle delay in cells seeded on pre-irradiated Mylar® compared to untreated Mylar®. It should be noted that percent of cells in G0/G1 phase shifts when cells are exposed to additional radiation (Fig. 29) and cells seeded on untreated surfaces delayed in G0/G1 phase compared to cells seeded on pre-irradiated Mylar®. However, after 24 hours of incubation the cycle distribution of cells seeded both on untreated and pre-irradiated Mylar® becomes identical (Figs. 30, 31), so we can conclude that cell-cycle distribution returns to normal twenty four hours after irradiation. If we examine a 24 hours time-course of fraction of cells in G0/G1 phase we can see that irradiation of cells seeded on untreated Mylar® leads to dose-dependent increase in number of cells accumulated in G0/G1 phase immediately after irradiation (Fig. 32). Cells seeded on pre-irradiated with 2 Gy of alpha particles Mylar® do not demonstrate a dose-dependent accumulation of cells in G0/G1 phase. Additional exposure to ionizing radiation of cells seeded on pre-irradiated Mylar® seems to have a reverse effect (Fig. 33). Nevertheless, any effect of irradiation is cleared after 8 hours of incubation and percent of cells in G0/G1 phase become virtually the same in all cases (Figs. 32, 33).

According to our observations there is a slight cell-cycle delay in irradiated cells and in cells seeded on pre-irradiated surfaces, but the delay is not significant and disappears within next 8 hours. Therefore, we conclude that the cell-cycle delay does not have a significant influence on micronucleus assay and the micronucleus assay can be performed for qualitative analysis of radiation influence on CHO cell.

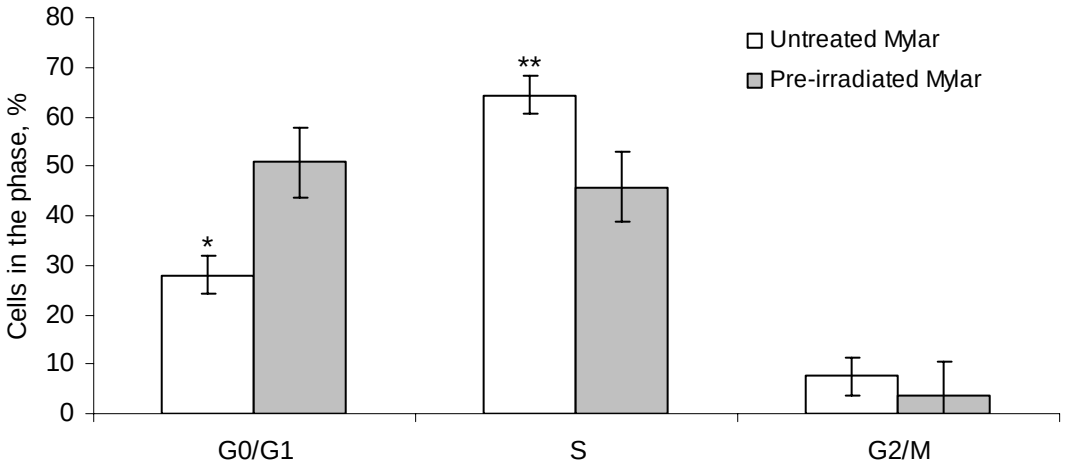

Fig. 28. Cell-cycle distribution of un-irradiated cells 48 hours after seeding. Cells were seeded on untreated (open bars) or pre-irradiated with 2 Gy of alpha particles (shaded bars) Mylar®-bottomed dishes. 48 Hours later cells were fixed and flow cytometry analysis was performed. Error bars represent the coefficient of variation. Significantly different data for each point marked (*) or (**). $P_{value} < 0.0005$.

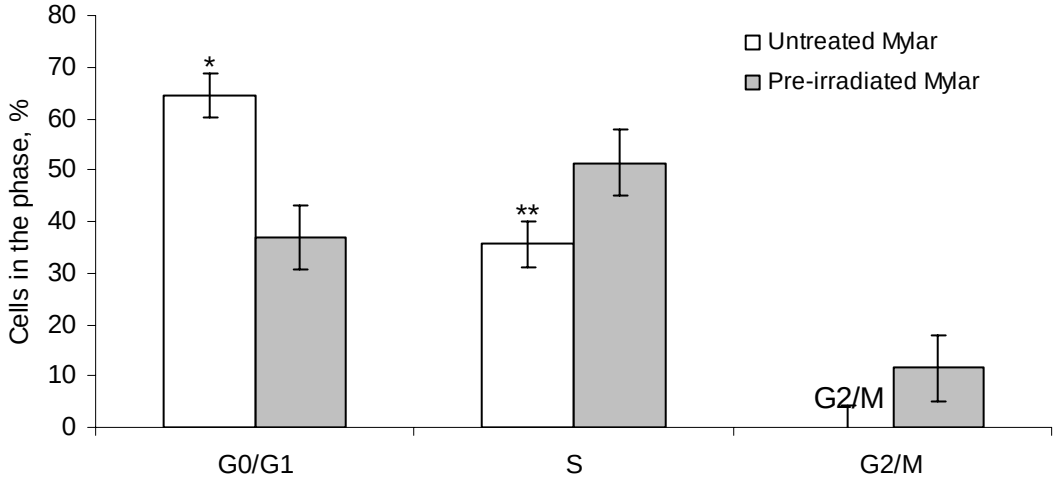

Fig. 29. Cell-cycle distribution of cells immediately after exposure to high-LET radiation. Cells were seeded on untreated (open bars) or pre-irradiated with 2 Gy of alpha-particles (shaded bars) Mylar®-bottomed dishes. 48 Hours later cells were exposed to 4 Gy of alpha particles. Immediately after that cells were fixed and flow cytometry analysis was performed. Error bars represent the coefficient of variation. Significantly different data for each point marked (*) or (**). $P_{value} < 0.0005$.

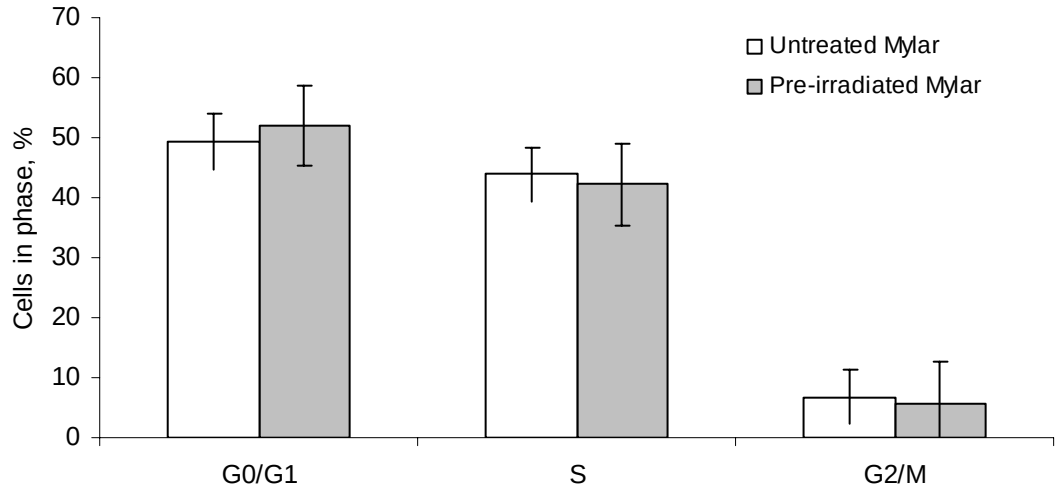

Fig. 30. Cell-cycle distribution of un-irradiated cells 72 hours after seeding. Cells were seeded on untreated (open bars) or pre-irradiated with 2 Gy of alpha particles (shaded bars) Mylar®-bottomed dishes. 72 Hours later cells were fixed and flow cytometry analysis was performed. Error bars represent the coefficient of variation.

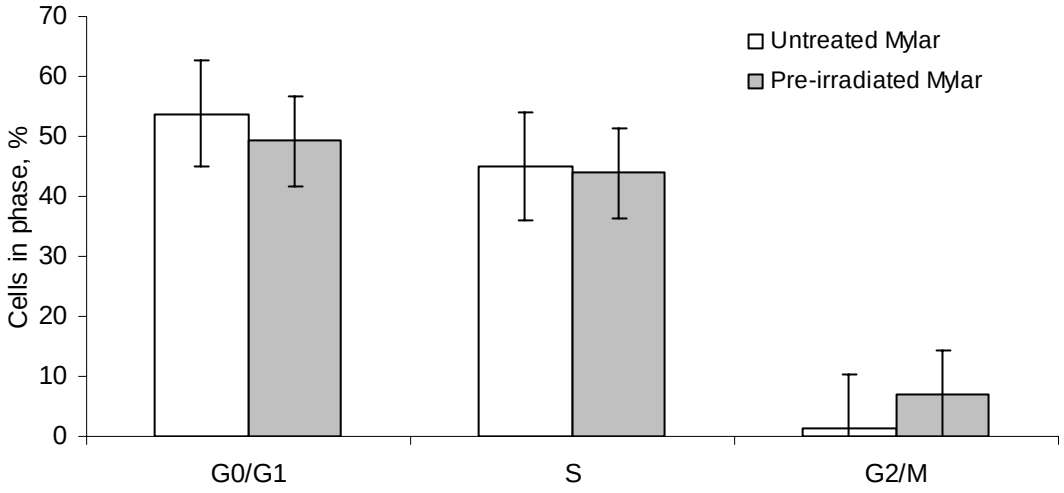

Fig. 31. Cell-cycle distribution of cells 24 hours after exposure to high-LET radiation. Cells were seeded on untreated (open bars) or pre-irradiated with 2 Gy of alpha particles (shaded bars) Mylar®-bottomed dishes. 48 Hours later cells were exposed to 4 Gy of alpha particles and incubated for 24 hours. Then cells were fixed and flow cytometry analysis was performed. Error bars represent the coefficient of variation.

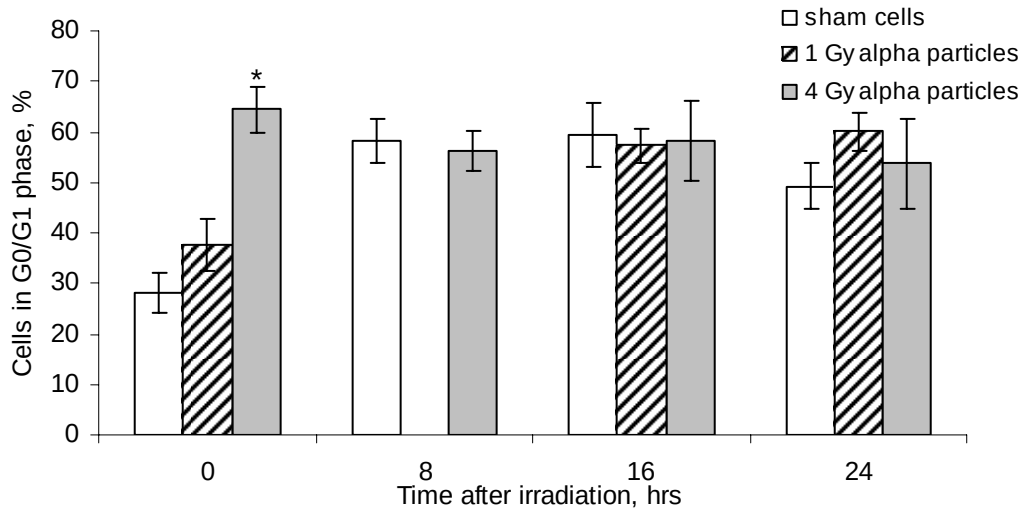

Fig. 32. Twenty four hours time-course of cells in G0/G1 phase seeded on untreated Mylar®. Cells were seeded on untreated Mylar®. 48 Hours later cells were exposed to 1 Gy (hatched bars), or 4 Gy (shaded bars) of alpha radiation, or left un-irradiated as a control (open bars). Error bars represent the coefficient of variation. Significantly different data for each point marked (*). $P_{value} < 0.0005$.

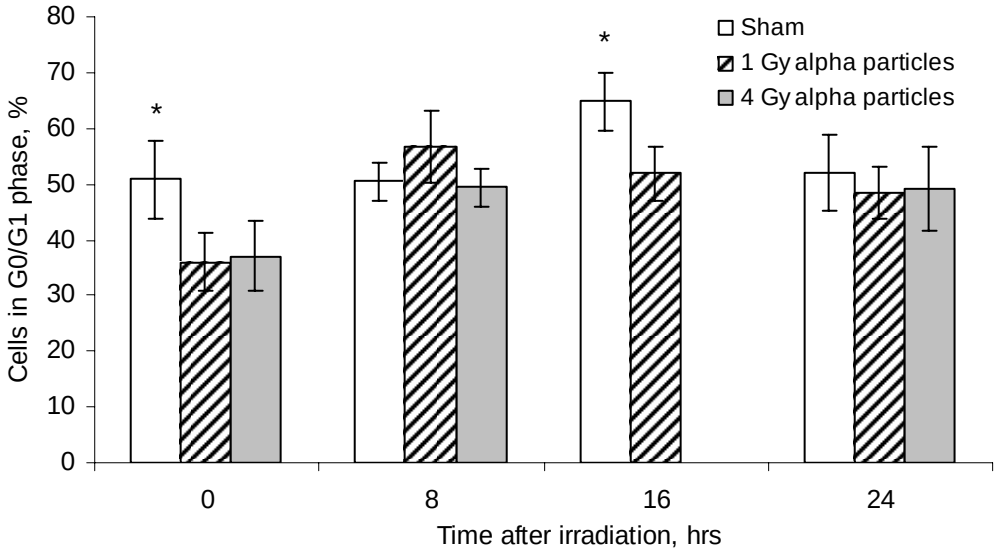

Fig. 33. Twenty four hours time-course of cells in G0/G1 phase seeded on pre-irradiated Mylar®. Cells were seeded on pre-irradiated with 2 Gy of alpha particles Mylar®. 48 Hours later cells were exposed to 1 Gy (hatched bars), or 4 Gy (shaded bars) of alpha radiation, or left un-irradiated as a control (open bars). Error bars represent the coefficient of variation. Significantly different data for each point marked (*). $P_{value} \leq 0.02$.

In order to study the possible impact of irradiated nutrient medium, cells were seeded into the medium preirradiated with 2 Gy of X rays or alpha particles in Mylar®-bottomed vessels and glass-bottomed tissue-culture plastic chambers.

Figure 34 depicts the micronucleus frequency in cells seeded into untreated medium on Mylar® dishes (Mylar-1) and into preirradiated with 2 Gy of alpha particles medium (Mylar-2). The micronucleus frequency significantly increases in cells seeded into preirradiated medium (data for 0 Gy; P_{value}=0.03). The micronucleus frequency also stays significantly elevated after irradiation of cells seeded into pre-irradiated medium with 1 Gy of alpha particles (P_{value}<0.0005). However, irradiation of cells growing in pre-irradiated medium with doses 2 Gy and higher seems not to impact frequency of micronuclei in these cells. The general picture shows that the micronucleus frequency gradually increases with dose in cells seeded into untreated medium as well as in cells seeded in pre-irradiated medium, except that micronucleus frequency versus dose relationship in cells exposed to radiation in pre-irradiated medium is less steep.

Pre-irradiation of nutrient medium in Mylar® dishes with X rays (Fig. 35) led to abrupt increase of micronucleus frequency (about 50% compared to micronucleus frequency in cells seeded in untreated medium; P_{value}<0.005), but further exposure to relatively low doses (up to 2 Gy) of ionizing radiation did not cause significant changes in micronucleus frequency. Exposure of cells seeded in medium pre-irradiated with high doses of X rays led to increase of micronucleus frequency up to 20% compared to cells irradiated in untreated medium.

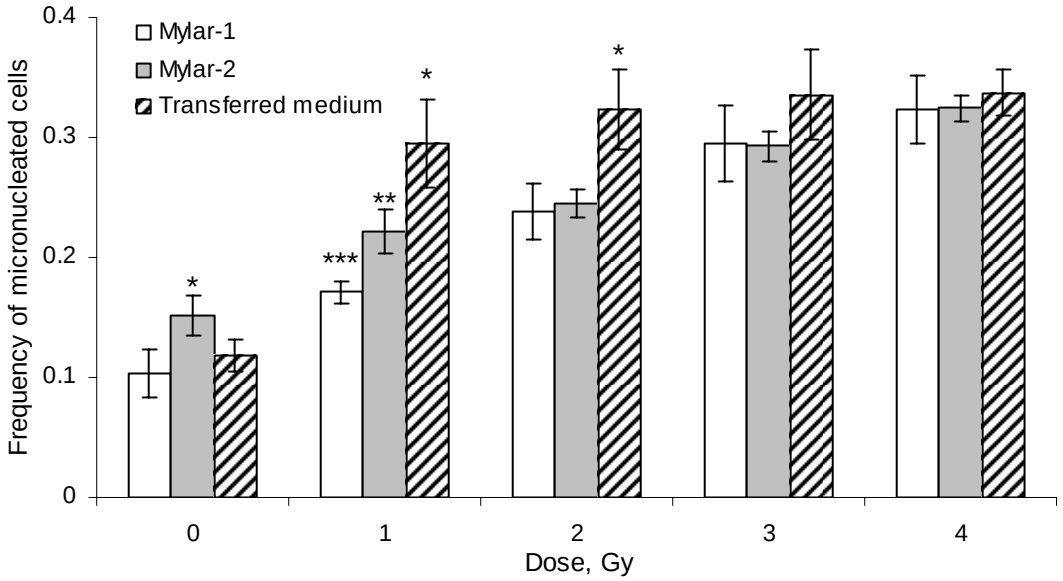

Fig. 34. Micronucleus frequency of cells exposed to high-LET radiation on Mylar®. CHO cells were seeded in nutrient medium which was un-irradiated (open bars) or previously irradiated (shaded bars) with 2 Gy of alpha particles in Mylar®-bottomed dishes and in pre-irradiated with 2 Gy of alpha particles medium transferred into fresh Mylar® -bot dishes (hatched bars). Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each point is labelled with (*), (**) and (***). $P_{value} \leq 0.03$.

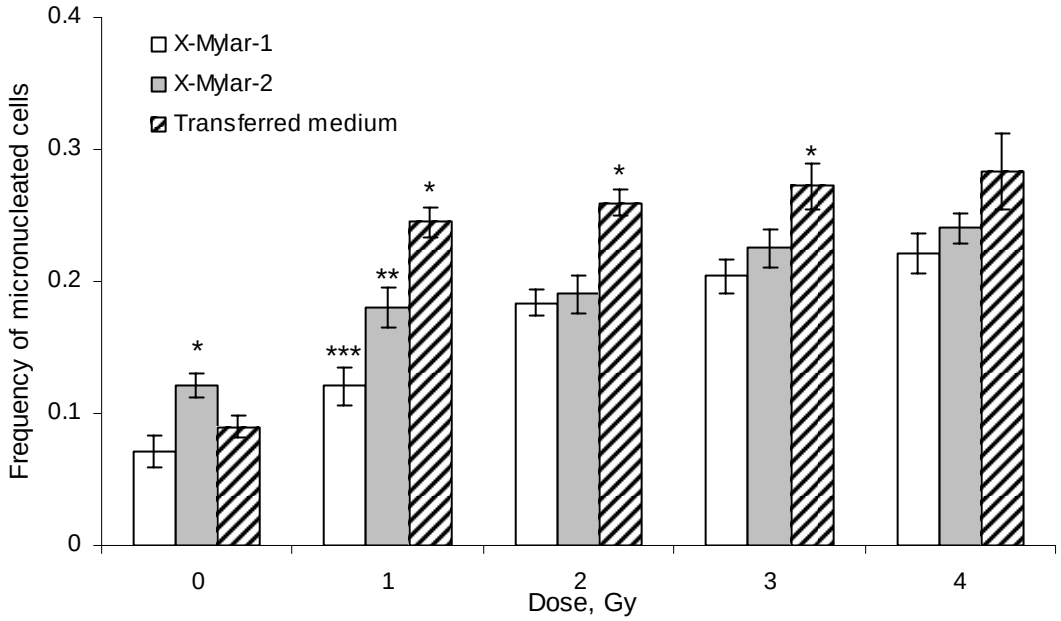

Fig. 35. Micronucleus frequency of cells exposed to low-LET radiation on Mylar®. CHO cells were seeded in nutrient medium which was un-irradiated (open bars) or previously irradiated (shaded bars) with 2 Gy of X rays in Mylar®-bottomed dishes and in pre-irradiated with 2 Gy of X rays medium transferred into fresh Mylar®-bottomed dishes (hatched bars). Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each point is labelled with (*), (**) and (***). $P_{value} \leq 0.01$.

The trend is the same in case of medium pre-irradiated in glass-bottomed tissue-culture plastic chambers (Fig. 36). Significant increase of micronucleus frequency (P_{value}=0.04) in cells seeded in pre-irradiated medium compared to that in cells seeded in untreated medium. Virtually no change in micronucleus frequency was observed when cells were exposed to 1 to 4 Gy of X rays (Fig. 36).

In order to study effects from pre-irradiated nutrient medium more clearly, medium was transferred from some of Mylar® dishes and glass-bottomed chambers culture vessels immediately after pre-irradiation and the experiment was repeated with transferred medium. As can be seen, micronucleus frequency of cells seeded in transferred medium is virtually the same as micronucleus frequency of cells seeded in untreated medium (Figs. 34 – 36, data for 0 Gy). However, further exposure with ionizing radiation resulted in drastic increase of micronucleus frequency in cells seeded in pre-irradiated medium ($P_{value}\leq0.03$, data for 1 and 2 Gy), especially in glass-bottomed chambers. It should be noted that elevated micronucleus frequency of cells irradiated in transferred medium does not vary considerably with the dose of ionizing radiation.

When we compare the micronucleus frequency in cells seeded on the pre-irradiated surfaces and the micronucleus frequency of cells seeded in irradiated medium, there is no significant difference (Figs. 37 – 39). The micronucleus frequency of cells which were in contact with pre-irradiated substrate (pre-irradiated surface or irradiated nutrient medium) is up to 25% (P_{value}=0.02) higher than the micronucleus frequency of cells which were in contact with untreated substrate in case of the

experiment with alpha particles on Mylar® (Fig. 37, data for 0 Gy) and in case of the experiment with X rays on glass-bottomed chambers (Fig. 39, data for 0 Gy, $P_{value}<0.0005$), and up to 50% higher in case of the experiment with X rays on Mylar® (Fig. 38, data for 0 Gy, $P_{value}<0.0005$). The micronucleus frequency remains elevated compared to controls in cells irradiated with 1 Gy on pre-irradiated with either alpha particles or X rays Mylar® (up to 30%, $P_{value}<0.0005$) (Figs. 37, 38), but additional irradiation of cells which were in contact with pre-irradiated glass-bottomed chamber does not lead to noticeable changes micronucleus frequency (Fig. 39).

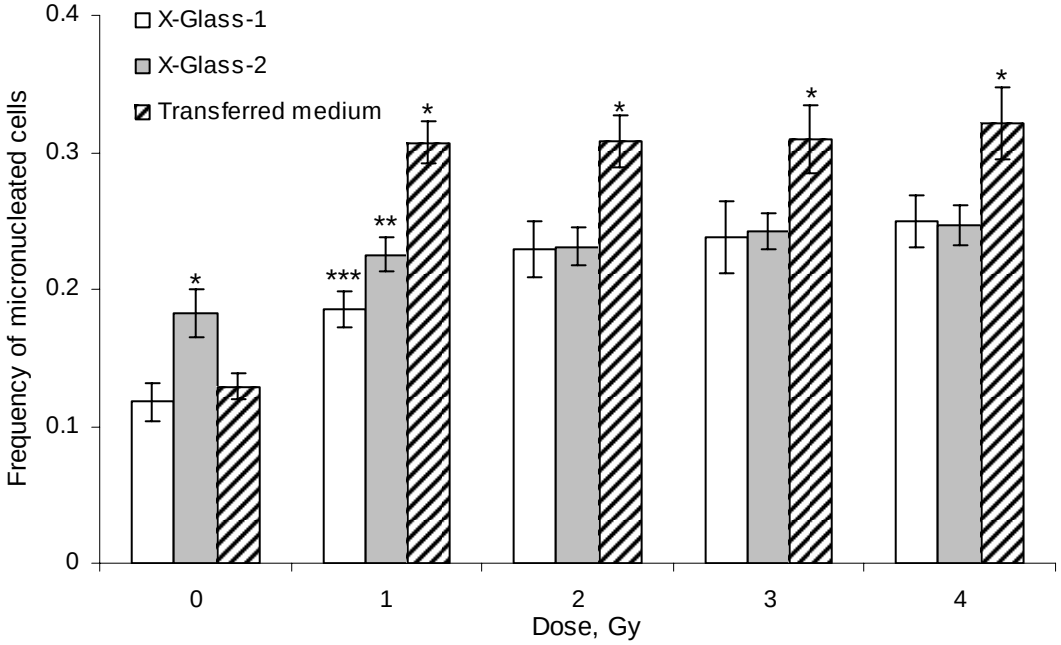

Fig. 36. Micronucleus frequency of cells exposed to low-LET radiation on tissue culture chambers. CHO cells were seeded in nutrient medium which was un-irradiated (open bars) or previously irradiated (shaded bars) with 2 Gy of X rays in glass-bottomed tissue culture plastic chambers and in pre-irradiated with 2 Gy of X rays medium transferred into fresh glass-bottome tissue culture chambers (hatched bars). Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each point is labelled with (*), (**) and (***). P_{value} is < 0.0005.

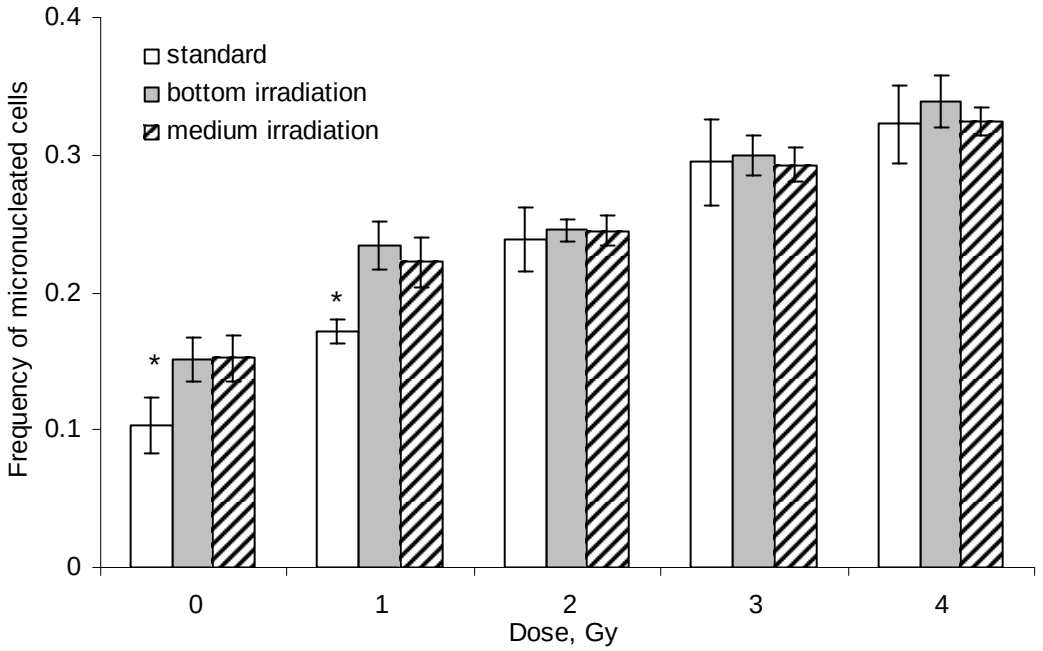

Fig. 37. Micronucleus frequency of cells exposed to high-LET radiation on Mylar® under different conditions. Cells were seeded on untreated Mylar® (open bars), or previously irradiated with 2 Gy of alpha particles Mylar® (shaded bars), or in pre-irradiated inside the Mylar® dishes with 2 Gy of alpha particles nutrient medium (hatched bars). Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each point is labelled with (*). $P_{value} < 0.005$.

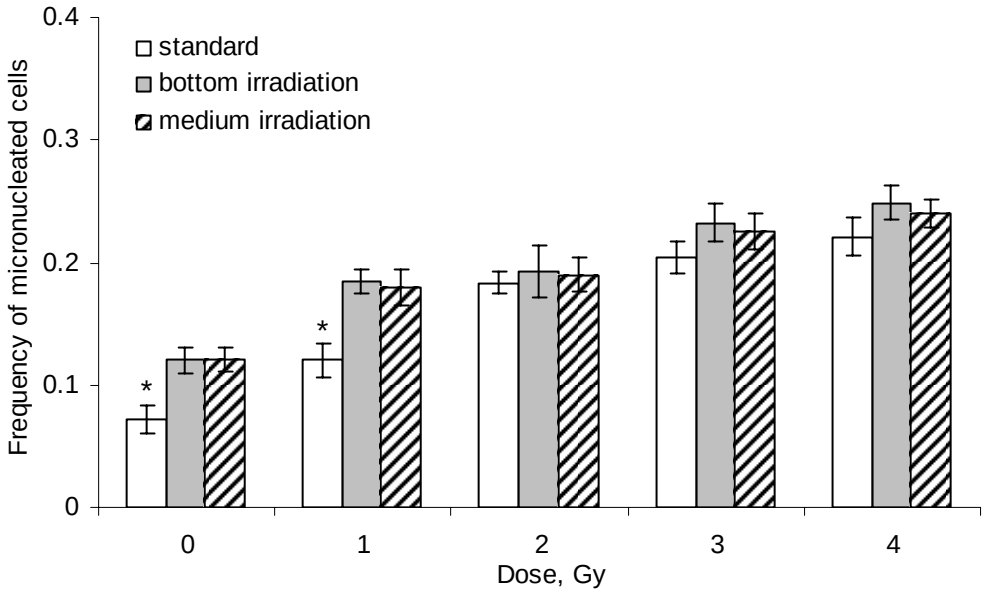

Fig. 38 Micronucleus frequency of cells exposed to low-LET radiation on Mylar® under different conditions. Cells were seeded on untreated Mylar® (open bars), or previously irradiated with 2 Gy of X rays Mylar® (shaded bars), or in pre-irradiated inside the Mylar® dishes with 2 Gy of X rays nutrient medium (hatched bars). Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each point is labelled with (*). $P_{value} < 0.0005$.

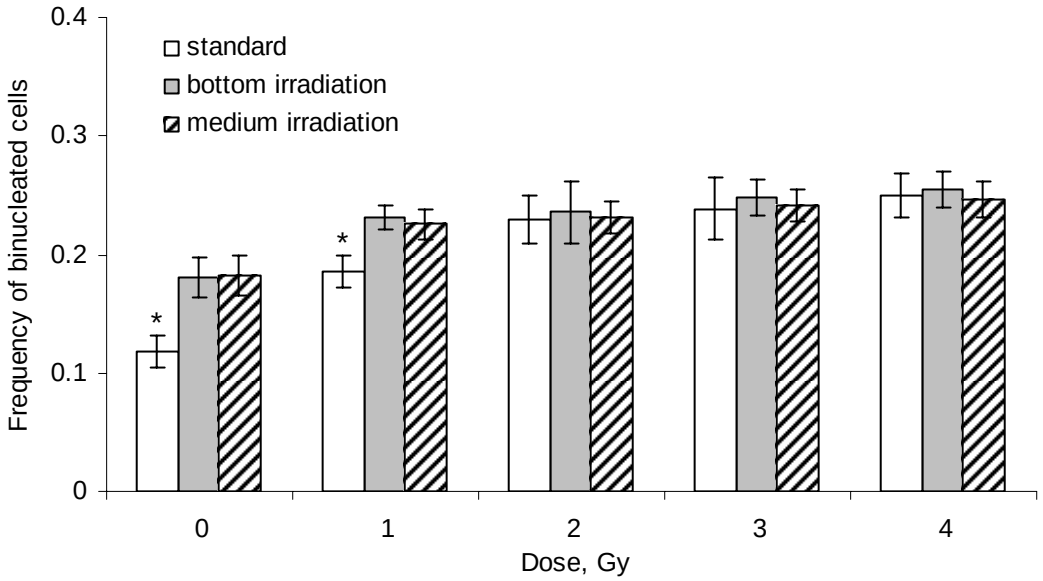

Fig. 39. Micronucleus frequency of cells exposed to low-LET radiation on tissue culture chambers under different conditions. Cells were seeded on untreated chambers (open bars), or previously irradiated with 2 Gy of X rays chambers (shaded bars), or in pre-irradiated inside the chambers with 2 Gy of X rays nutrient medium (hatched bars). Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each point is labelled with (*). $P_{value} < 0.0005$.

Discussion

The results of our previous experiments demonstrated that pre-irradiation of cell culture vessels leads to abrupt increase of micronucleus frequency in cells seeded then on pre-irradiated surfaces, which led us to conclusion that the material of cell culture vessels changes its properties after exposure to ionizing radiation.

The present results confirm that even a moderate dose (2 Gy) of ionizing radiation alters properties of the nutrient medium and the material of cell culture vessels resulting in elevated micronucleus frequency in cells growing in contact with pre-irradiated material.

In Figure 36, the micronucleus frequency of cells exposed to X rays in pre-irradiated medium in glass-bottomed tissue-culture chambers does not vary considerably from micronucleus frequency of cells irradiated in untreated medium in the same kind of chambers. At the same time cells X-ray-irradiated in pre-irradiated medium in Mylar® dishes produce up to 20% ($P_{value}<0.005$) more micronuclei than cells irradiated on Mylar® in untreated medium (Fig. 35). The frequency of micronucleated cells increases gradually with dose both in case of X-ray-irradiation and alpha-irradiation, except this trend is less obvious in case of cells plated in pre-irradiated medium in glass-bottomed chambers.

The micronucleus frequency of cells seeded on pre-irradiated surfaces and micronucleus frequency of cells seeded into pre-irradiated medium (Figs. 37 – 39) is virtually the same. But it should be noted that medium was pre-irradiated inside the culture vessels, therefore it is hard to distinguish whether the effect was due to surface

exposure to ionizing radiation or medium exposure. Based on the following observations we may conclude that there are some indications on interaction between irradiated material of culture vessels and nutrient medium. While cells seeded in pre-irradiated medium demonstrate increased micronucleation compared to cells seeded in untreated medium, cells growing in medium which was transferred immediately after irradiation into untreated vessel have nearly the same micronucleus frequency as cells growing in untreated medium (Figs. 34–36, data for 0 Gy). When we take into account the fact that the micronucleus frequency in cells seeded into transferred medium increased significantly after the exposure of cells to ionizing radiation and there is virtually no change in the micronucleus frequency of cells seeded in transferred medium, but not irradiated (Figs. 34 – 36), we conclude that the major influence on cell micronucleation is mainly due to surface effects. If there are volatile chemicals trapped in the structure of material (59), we suggest that influence of even low doses of radiation leads to release of these compounds. Since fresh nutrient medium is essentially a buffer solution containing antioxidants, it can relieve the consequences of first irradiation (when the culture vessel was pre-irradiated with medium inside). But once irradiated, medium may not be such a good protectant, so we can observe the effect of drastic increase of micronucleus frequency in transferred to the fresh culture vessel and again exposed to ionizing radiation medium.

If we compare the micronucleus frequency of cells exposed to ionizing radiation in pre-irradiated medium in different containers (Fig. 40), it can be clearly seen that the level of micronucleation depends more on the type of cell culture vessel than on type or

dose of ionizing radiation especially for doses from 1 to 3 Gy. For the lowest dose examined (0.5 Gy) there is no apparent change in micronucleus frequency compared to the micronucleus frequency of un-irradiated cells seeded in pre-irradiated medium. The micronucleus frequency of cells exposed to the highest dose of radiation (4 Gy) started to demonstrate radiation-type dependence. The same trend can be observed in Figure 41, which depicts the micronucleus frequency of cells seeded on pre-irradiated surfaces. The micronucleus frequency of cells seeded on pre-irradiated with X rays glass-bottomed tissue culture chambers and exposed then to moderate doses of X rays (1 or 2 Gy) is virtually the same as the micronucleus frequency of alpha-irradiated cells seeded on Mylar®-bottomed dishes which show obvious type-of-material dependent effect. When cells seeded on pre-irradiated surfaces were exposed to higher doses of ionizing radiation (3 or 4 Gy) the effect from type of radiation dominated and the micronucleus frequency of cells exposed to alpha-radiation increased significantly ($P_{value} \leq 0.04$). The micronucleus frequency of cells exposed to ionizing radiation on different untreated surfaces demonstrate more clear dependence on type of the material on which cells were plated (Fig. 42). At moderate doses of ionizing radiation micronucleus frequency of cells irradiated with X rays on glass-bottomed tissue-culture chambers either higher or equal to micronucleus frequency of cells exposed to alpha radiation on Mylar®-bottomed dishes. Therefore, we can clearly distinguish 3 major regions on the dose scale:

- Low-dose region, which is up to 1 Gy. This region is characterized by low dependence from additional exposure to ionizing radiation, but significant dependence on pre-treatment of culture vessel
- Moderate-dose region, which is from 1 up to 3 Gy. This region is characterized by strong dependence of micronucleus formation from the material of the cell culture vessel, low dose-dependence and low type of radiation dependence
- High-dose region, which is from 3 to 4 Gy (and, probably, higher). This region is characterized by dominated type of radiation dependence on micronucleus formation.

The differences in factors which influence micronucleus frequency in these regions suggest the difference in mechanisms responsible for cell damage could play an important role in experiment planning.

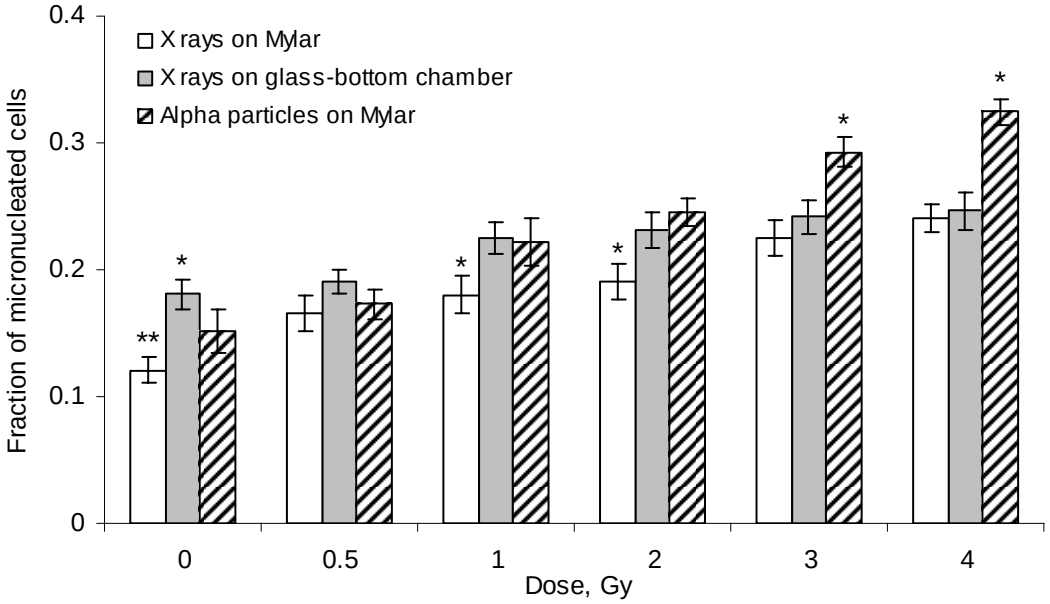

Fig. 40. Micronucleus frequency of cells seeded into pre-irradiated nutrient medium. Cells were plated in pre-irradiated with 2 Gy of X rays medium on Mylar®-bottomed dishes (open bars), or in pre-irradiated with 2 Gy of X rays medium on glass-bottomed tissue-culture plastic chambers (shaded bars), or in pre-irradiated with 2 Gy of alpha particles medium on Mylar® -bott dishes (hatched bars). 48 Hours later cells were exposed to various doses of X rays or alpha particles. Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each point is labelled with (*) or (**). $P_{value} \leq 0.046$.

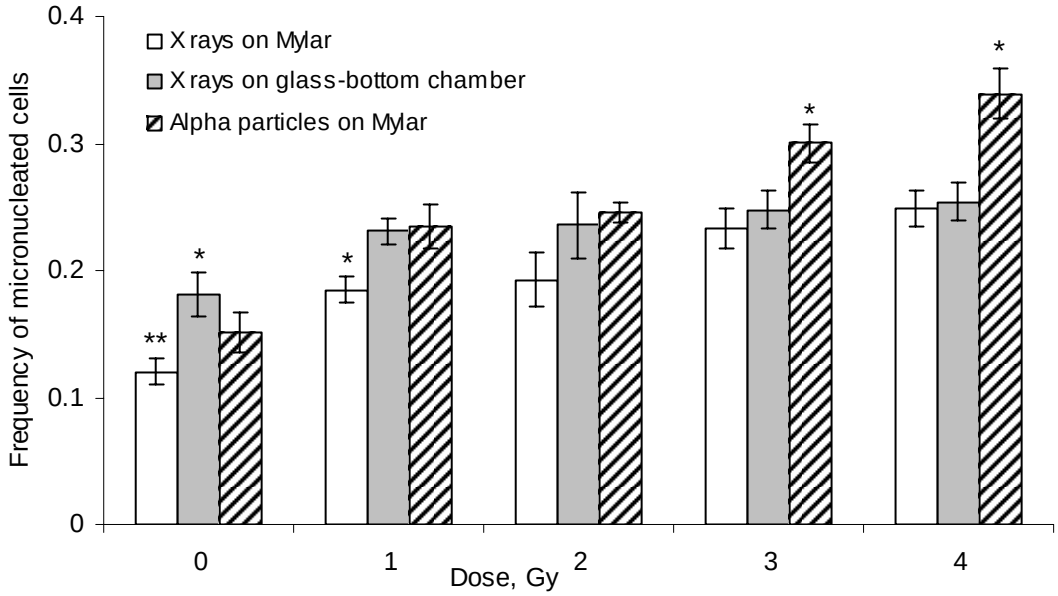

Fig. 41. Micronucleus frequency of cells seeded on pre-irradiated surfaces. Cells were plated on pre-irradiated with 2 Gy of X rays Mylar®-bottomed dishes (open bars), or on pre-irradiated with 2 Gy of X rays glass-bottomed tissue-culture plastic chambers (shaded bars), or on pre-irradiated with 2 Gy of alpha particles Mylar®-bottomed dishes (hatched bars). 48 Hours later cells were exposed to various doses of X rays or alpha particles. Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments.Significantly different data for each point is labelled with (*) or (**). $P_{value} \leq 0.03$.

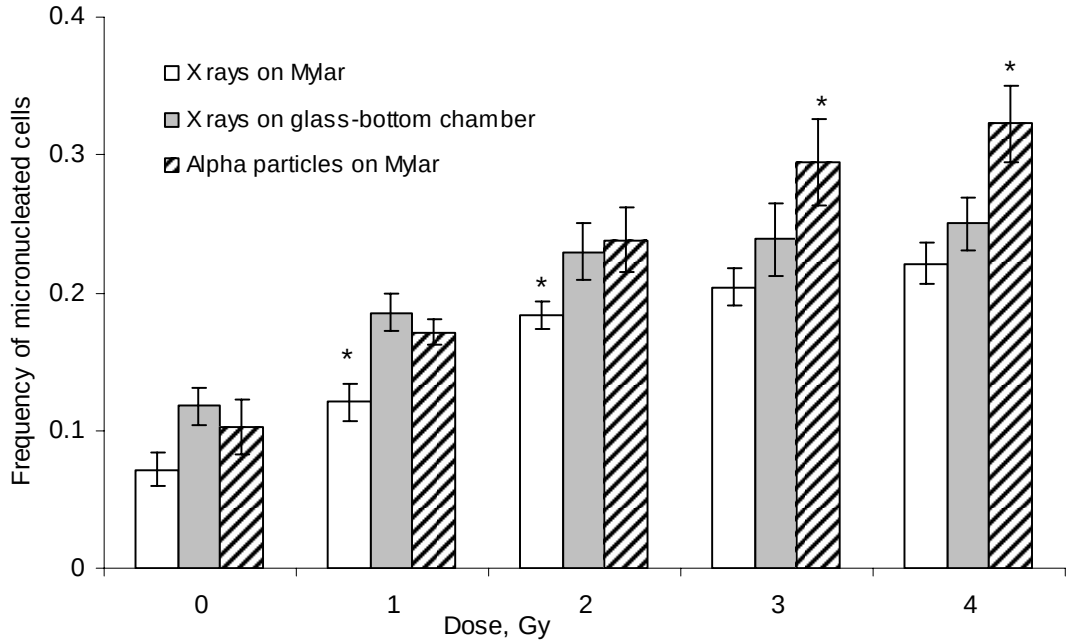

Fig. 42. Micronucleus frequency of cells seeded on untreated surfaces. Cells were plated on Mylar®-bottomed dishes and 48 hours later irradiated with various doses of X rays (open bars), or on glass-bottomed tissue-culture plastic chambers and 48 hours later irradiated with various doses of X rays (shaded bars), or on Mylar®-bottomed dishes and 48 hours later irradiated with various doses of alpha particles (hatched bars). Error bars represent standard deviation of the mean from combined experiments, consisting of three replicate samples each from two separate experiments. Significantly different data for each point is labelled with (*). $P_{value} \leq 0.04$.

CHAPTER V

SUMMARY AND CONCLUSIONS

The present study applied the micronucleus assay as a tool for assessment of the clastogenic effects of different types of radiation in Chinese Hamster Ovary (CHO) cells. During the study the basic physical characteristics of micronuclei and nuclei such as size and intensity of DAPI fluorescence (Figs. 2 – 6), dependence of micronucleus formation on dose and type of radiation (Fig. 8) were investigated.

Also, our experiments confirmed that CHO cells do not undergo apoptotic mode of death after exposure to ionizing radiation (47, 48), but form micronuclei.

The persistence of radiation-induced micronuclei was investigated and found that the frequency remains significantly elevated up to twenty four hours after irradiation (Figs. 35, 36). It has been established that micronucleated cells influence neighbor cells leading to prolongation of an elevated micronucleus frequency in the mixture of irradiated cells and untreated cells (up to five weeks), which is probably due to increased formation of micronuclei by neighbor cells (Figs. 38, 39).

Since irradiation of cells growing in a cell culture vessels is an essential part of many *in vitro* experiments, the study of interactions of noncellular matrix (cell-culture vessels and nutrient medium) with cells in culture was performed. The influence of pre-irradiation of cell-culture surfaces was studied utilizing coverslip culture chambers and Mylar®-bottomed dishes. A critical observation was that the effect of radiation on the culture surfaces has a marked effect on micronucleus frequency.

The elevated micronucleus frequency in cells seeded in glass-bottomed plastic containers has been noted in all our experiments as well as insignificant changes in micronucleation with dose of ionizing radiation. This observation coupled with one that cells seeded on glass surfaces demonstrated a lower plating efficiency led us to the conclusion that the material of glass-bottomed tissue-culture chambers undergoes some chemical changes under the influence of ionizing radiation and, therefore, is less appropriate for radiation experiments with CHO cells than Mylar-bottomed dishes. According to several studies (59, 60) plastics sterilized with high-dose radiation have numerous volatile radiolysis products such as benzene trapped inside the structure which release may affect the viability of cells growing in those chambers.

The most distinctive results were obtained when pre-irradiated medium was transferred into new culture vessels of the same type and cells were seeded in those vessels. While micronucleus frequency of cells plated in transferred medium was virtually the same as micronucleus frequency of controls (Figs. 24 – 36, data for 0 Gy), exposure of cells seeded in transferred medium to ionizing radiation resulted in highly elevated micronucleus frequency which did not vary significantly with dose of applied radiation. Once again, the most drastic changes in cell micronucleation were registered at the lowest doses (up to 2 Gy). Taking into account the fact that the material of cell culture vessels may contain some volatile products, it is possible that exposure of the vessels to ionizing radiation disengage those products from the polymer structure into nutrient medium. Obviously, secondary irradiation of the same vessel does not lead to the same result, but when pre-irradiated medium is transferred to a new vessel and

irradiation performed again, additional clastogenic compounds are released into medium. Therefore the reason for such a peculiar behavior of cells irradiated in transferred into new vessels could be a double dose of liberated chemicals by the nutrient medium. The fact that the elevated micronucleus frequency of those cells virtually does not depend on dose of applied radiation means that the influence of irradiated polymer is greater than the impact of radiation itself. As can be seen, the micronucleus frequency of the cells exposed to high doses of alpha radiation in transferred medium (Fig. 34, data for 4 Gy) is the same as the micronucleus frequency of controls, while the micronucleus frequency of X-ray-irradiated cells (Fig. 35, data for 4 Gy) is higher then micronucleus frequency of controls. The results of the experiment with cells seeded on glass-bottomed chambers support this hypothesis. The micronucleus frequency of cells seeded on these vessels remains significantly elevated compared to micronucleus frequency of cells seeded on Mylar®-bottomed dishes under the same conditions. Results of the experiments performed by other group (76) support the observation that cells seeded on pre-irradiated tissue culture plastics demonstrate an unusual number of abnormalities compared to cells seeded on untreated surfaces.

All these observations lead us to conclusion that there are several mechanisms responsible for damage of cells exposed *in vitro*, and these mechanisms depend on dose level, type of radiation and, generally, on the type of material chosen for the radiation experiments in culture. In our experiments Mylar®-bottomed dishes demonstrated much better performance than glass-bottom tissue culture plastic chambers. Experiments with cells seeded and exposed to X ray radiation on the glass-bottomed plastic chambers

resulted in micronucleus yield compared to one of cells alpha-irradiated on Mylar®-bottomed dishes, while the micronucleus frequency of cells X-ray-irradiated on Mylar® was significantly lower according to relative biological effectiveness of alpha particles and X rays. Therefore, we may conclude that cell culture containers composed from plastic should be avoided in radiation experiments with Chinese hamster ovary cells.

Even though Mylar® film looks more suitable than borosilicate glass bottomed chambers with tissue-culture plastic walls, the best solution may be to identify more radiation-resistant films.

In order to identify the chemical species that might be responsible for these effects additional experiments should be performed. The general method for determination of volatile chemical compounds in materials is by thermal desorption-gas chromatography-mass spectroscopy. Utilizing this method we could possibly identify traces of such compounds as aliphatic hydrocarbons, methylketones, degradation products of antioxidants, and many cyclic compounds such as benzene, which have been found in polymers in significant doses (59).

While the micronucleus assay is a very useful tool in biological damage assessment, the micronucleus yield is dependent on many variables. The results should always be interpreted with some caution as some cell types are sensitive to the effects of radiation on their surroundings. It may be quite difficult to differentiate effects due to direct cell irradiation those due to radiation-damaged environment or bystander effects. The non-cellular matrix irradiation effects could magnify the effects observed in very low dose experiments used to examine the bystander effect and at the very least is a

confounding factor in determining the magnitude and degree of toxicity that can be assigned to a bystander effect.

REFERENCES

1. I. A. Alov and V. V. Kazan'ev, Fate of lagging chromosomes and micronuclei. *Dokl. Akad. Nauk SSSR* **187**, 191 – 192 (1969).

2. J. R. K. Savage, A comment on the quantitative relationship between micronuclei and chromosome aberration, *Mutat. Res*. **207**, 33 – 36 (1988).

3. F. D. Solomon, P. P. Venkatachalam and R. K. Jeevanram, Analysis of radiation dose-response curve obtained with cytokinesis-block micronuclei assay, *Nucl. Med. Biol.* **24** 413 – 416 (1997).

4. M. J. Ramirez, J. Surralles, S. Puerto, A. Creus and R. Marcos, Low persistence of radiation-induced centromeres positive and negative micronuclei in cultured human cells. *Mutat. Res*. **440**, 163 – 169 (1999).

5. W. P. Chang, M. S. Tsai, J. S. Hwang, Y. P. Lin, W. A. Hsieh and S. Y. Huang, Follow-up in the micronucleus frequencies and its subsets in human population with chronic low-dose gamma-irradiation exposure. *Mutat Res*. **428**, 99 – 105 (1999).

6. M. Abend, S. Frombeckand, and D. van Beuningen, Indications for an active process underlying spontaneous and radiation-induced micronucleation in L929 cells. *Int.J. Radiat. Biol*. **75**, 1567 – 1578 (1999).

7. N. Sato, K. Mizumoto M. Nakamura and M. Tanaka, Radiation-induced centrosome overduplication and multiple mitotic spindles in human tumor cells. *Exp. Cell Res*. **255**, 321 – 326 (2000).

8. I. Ponsa, J. F. Barquinero, R. Miro, J. Egozcue and A. Genesca, Nondisjunction and chromosome loss in gamma-irradiated human lymphocytes: A fluorescence *in situ* hybridization analysis using centromeres-specific probes. ***Radiat. Res.*** **155**, 424 – 431 (2001).

9. K. J. Sorensen, L. A. Zetterberg, D. O. Nelson, J. Grawe and J. D. Tucker, The *in vivo* dose rate effect of chronic gamma radiation in mice: Translocation and micronucleus analyses. ***Mutat. Res***. **457**, 125 – 136 (2000).

10. *ICRP Recommendations of the International Commission on Radiological Protection*, ICRP Publication 60, Pergamon Press, Oxford, 1991.

11. D. R Boreham, J. A. Dolling, S. R. Maves, N. Siwarungsun and R. E. Mitchel, Dose-rate effects for apoptosis and micronucleus formation in gamma-irradiated human lymphocytes, ***Radiat. Res***. **153**, 579 – 586 (2000).

12. A. Sgura, A. Antoccia, R. Cherubini and C. Tanzarella, Chromosome nondisjunction and loss induced by protons and X-rays in primary human fibroblasts: Role of centromeres in aneuploidy. *Radiat Res*. **156**, 225 – 231 (2001).

13. K. M. Prise, M. Folkard, A. M. Malcolmson, C. H. Pullar, G. Schettino, A. G. Bowey and B. D. Michael, Single ion actions: The induction of micronuclei in V79 cells exposed to individual protons. ***Adv. Space Res***. **25**, 2095 – 2101 (2000).

14. Y. Yamada, Y. Oghiso, H. Enomoto and N. Ishigure, Induction of micronuclei in a rat alveolar epithelial cell line by alpha particle irradiation. ***Radiat. Prot. Dosimetry*** **99,** 219-222 (2002).

15. M. Fenech and A. A. Morley, Measurement of micronuclei in lymphocytes. *Mutat. Res*. **147**, 29 – 36 (1985).

16. B. E. Peace, G. Livingston, E. B. Silberstein and J. C. Loper, A case of elevated spontaneous micronucleus frequency derived from chromosome 2. *Mutat. Res*. **430**, 109 – 119 (1999).

17. S. Gutierrez, E. Carbonell, P. Galofre, A. Creus and R. Marcos, Cytogenic damage after 131-iodine treatment for hyperthyroidism and thyroid cancer. A study using the micronucleus test. *Europ. J. Nucl. Med*. **26**, 1589 – 1596 (1999).

18. G. C. Jagetia, A. Jayakrishnan, D. Fernandes and M. S. Vidyasagar, Evaluation of micronuclei frequency in the cultured peripheral blood lymphocytes of cancer patients before and after radiation treatment. *Mutat. Res*. **491**, 9 – 16 (2001).

19. N. L. Shmakova, T. A. Fedeeva, E. A. Nasonova, E. A. Krasavin and A. V. Rzianina, Cytogenetic effects of low doses of radiation in mammalian cells: Analysis of the hypersensitivity phenomenon and induced resistance. *Radiats. Biol. Radioecol*. **42,** 245 – 250 (2002).

20. W. F. Morgan, Non-targeted and delayed effects of exposure to ionizing radiation: I. Radiation-induced genomic instability and bystander effects *in vitro*. *Radiat. Res*. **159**, 567-580 (2003).

21. W. F. Morgan, Non-targeted and delayed effects of exposure to ionizing radiation: II. Radiation-induced genomic instability and bystander effects *in vivo*, clastogenic factors and transgenerational effects. *Radiat. Res*. **159**, 581-596 (2003).

22. F. Ballarini, M. Biaggi, A. Ottolenghi and O. Sapora, Cellular communication and bystander effects: A critical review for modeling low-dose radiation action. *Mutat. Res*. **501**, 1 – 12 (2002).

23. Z. Goldberg and B.E. Lehnert, Radiation-induced effects in unirradiated cells: A review and implications in cancer. *Int. J. Oncol.* **21**, 337 – 349 (2002).

24. J. Ostercher, K. M. Prise, B. D. Michael, J. Vogt, T. Butz and J. M. Tanner, Radiation-induced bystander effect mechanisms, biological implications and current investigations at the Leizig LIPSION facility. *Strahlenther Oncol*. **179**, 69 – 77 (2003).

25. T. Matsushima, M. Hayashi, A. Matsuoka, M. J. Ishidate, K. E. Miura, H. Shimizu, Y. Suzuki, K. Morimoto, H. Ogura, K. Mure, K. Koshi and T. Sofuni, Validation study of the *in vitro* micronucleus test in Chinese hamster lung cell line (CHL/IU). *Mutagenesis*. **14,** 569 – 580 (1999).

26. T. K. Lee, A. L. Wiley Jr, J. D. Esinhart and L. D. Blackburn, Radiation dose-dependent variations of micronuclei production in cytochalasin B – blocked human lymphocytes. *Teratog. Carcinog. Mutagen*. **14**, 1 – 12 (1994).

27. J. P. McNamee, P. V. Bellier, G. B. Gajda, S. M. Miller, E. P. Lemay, B. F. Lavalee and L. Marro, DNA damage and micronucleus induction in human leukocytes after acute *in vitro* exposure to a 1.9 GHz continuous-wave radiofrequency field. *Radiat. Res*. **158**, 523-533 (2002).

28. J. Y. Ma, H. M. Yang, M. W. Barger, P. D. Siegel, B. Z. Zhong, A. J. Kriech and V. Castranova, Alteration of pulmonary cytochrome p-450 system:

Effects of asphalt fume condensate exposure. *J. Toxicol. Environ. Health*. **65**, 1247 – 1260 (2002).

29. P. J. Johnston, E. Stoppard and P. E. Bryant, Induction and distribution of damage in CHO-K1 and the X-ray-sensitive hamster cell line *xrs5*, measured by the cytochalasin-B cytokinesis block micronucleus assay. *Mutat. Res*. **385**, 1 – 12 (1997).

30. C. Streffer, W.-U. Muller, A. Kryscio and W. Bocker, Micronuclei – Biological indicator for retrospective dosimetry after exposure to ionizing radiation. *Mutat. Res*. **404**, 101 – 105 (1998).

31. T. K. Lee, R. R. Allison, K. F. O'Brien, J. L. Naves, U. L. Karlsson and A. L. Wiley, Persistance of micronuclei in lymphocytes of cancer patients after radiotherapy. *Radiat. Res*. **157**, 678 – 684 (2002).

32. H. Thierens, A. Vral and L. de Ridder, Biological dosimetry using the micronucleus assay for lymphocytes: Interindividual differences in dose response. *Health Phys*. **61**, 623 – 630 (1991).

33. A. Sgura, A. Antoccia, R. Cherubini, M. D. Vecchia, P. Tiveron, F. Degrassi and C. Tanzarella, Micronuclei, CREST-positive micronuclei and cell inactivation induced in Chinese hamster cells by radiation with different quality. *Int. J. Radiat. Biol*. **76**, 367 – 374 (2000).

34. J. le Roux, J. Slabbert, B. Smit and G. Blekkenhorst, Assessment of the micronucleus assay as a biological dosimeter using cytokinesis-blocked lymphocytes from cancer patients receiving fractionated partial body radiotherapy. *Strahlenther Onkol*. **174**, 75 – 81 (1998).

35. N. Paillole and P. Voisin, Is micronuclei yield variability a problem for overexposure dose assessment to ionizing radiation? *Mutat. Res*. **413**, 47 – 56 (1998).

36. C. Mothersill and C. Seymour, Radiation-induced bystander effects: Past history and future directions. *Radiat. Res*. **155**, 759 – 767 (2001).

37. G. Schettino, M. Folkard, K. M. Prise, B. Vojnovic, K. D. Held and B. D. Michael, Low-dose studies of bystander cell killing with targeted soft X rays. *Radiat Res*. **160**, 505-511 (2003).

38. E. J. Broom, D. L. Brown and R. E. Mitchel, Dose responses for adaptation to low doses of Co^{60} γ-rays and H^3 β-particles in normal human fibroblasts. *Radiat. Res.* **158,** 181 – 186 (2002).

39. K. J. Sorensen, C. M. Attix, A. T. Christian, A. J. Wyrobek and J. D. Tucker, Adaptive response induction and variation in human lymphoblastoid cell lines. *Mutat. Res*. **519**, 15 – 24 (2002).

40. I. Lopez, A. Hilali, L. Versachaeve, C. Laurent, C. Bugueno, E. D. Leonard, A. Maes and P. Jadot, Influence of blood storage after *in vitro* exposure on the yield of micronuclei observed in human lymphocytes. *Strahlenther Oncol*. **169**, 258 – 262 (1993).

41. H. Jung, S. Y. Wang, I. W. Yang, D. W. Hsueh, W. J. Yang, T. H. Wang and H. S. Wang, Detection and treatment of mycoplasma contamination in cultured cells. *Chang Gung Med. J*. **26**, 250-258 (2003).

42. M. Fenech and A. A. Morley, Measurement of micronuclei in lymphocytes. *Mutat. Res*. **147**, 29 – 36 (1985).

43. M. Fenech, W. P. Chang, M. Kirsch-Volders, N. Holland, S. Bonassi and E. Zeiger, HUMN project: detailed description of the scoring criteria for the cytokinesis-block micronucleus assay using isolated human lymphocyte cultures. *Mutat. Res.* **534**, 65-75 (2003).

44. M. Fenech, The *in vitro* micronucleus technique. *Mutat Res* **455**, 81-95 (2000).

45. N. Wang, Y. Pan, T. Heiden and B. Tribukait. Fluorescence image cytometry for measurement of nuclear DNA content in surgical pathology. *Cytometry.* **22** 323 –329 (1995).

46. J. I. Epstein, W. N. Christensen, G. D. Steinberg and H. B. Carter Comparison of DNA ploidy and nuclear size, shape and chromatin irregularity in tissue sections and smears of prostatic carcinoma. *Anal Quant Cytol Histol*. **12** 352 – 358 (1990).

47. M. Abend, A. Rhein, K. P. Gilbertz, W. F. Blakely and D. Van Beuningen, Correlation of micronucleus and apoptosis assays with reproductive cell death. *Int. J. Radiat. Biol*. **67**, 315-326 (1995).

48. M. Abend, A. Rhein, K. P. Gilbertz and D. Van Beuningen, Evaluation of a modified micronucleus assay. *Int. J. Radiat. Biol*. **69**, 717-727 (1996).

49. I. F. Tannock and C. Lee, Evidence against apoptosis as a major mechanism for reproductive cell death following treatment of cell lines with anti-cancer drugs. *Br. J. Cancer*. **84**, 100-105 (2001).

50. T. Hu, C. M. Miller, G. M. Ridder and M. J. Aardema, Characterization of p53 in Chinese hamster cell lines CHO-K1, CHO-WBL, and CHL: Implications for genotoxicity testing. *Mutat Res*. **426**, 51-62 (1999).

51. C. Lindholm, H. Norppa, M. Hayashi and M. Sorsa, Induction of micronuclei and anaphase aberrations by cytochalasin B in human lymphocyte cultures. *Mutat. Res*. **260**, 369 – 375 (1991).

52. A. L. Brooks, G. J. Newton, L.-J. Shyr, F. A. Seiler and B. R. Scott, The combined effects of α-particles and X-rays on cell killing and micronuclei induction in lung epithelial cells. *Int. J. Radiat. Biol*. **58**, 799 – 811 (1990).

53. A. J. Mill, J. Wells, S. C. Hall and A. Butler, Micronucleus induction in human lymphocytes: comparative effects of X-rays, alpha particles, beta particles and neutrons and implications for biological dosimetry. *Radiat. Res*. **145**, 575 – 585 (1996).

54. M. J. Silva, A. Carothers, A. Dias, J. H. Luis, J. Piper and M. G. Boavida, Dose dependence of radiation-induced micronuclei in cytokinesis-blocked human lymphocytes. *Mutat. Res*. **322**, 117 – 128 (1994).

55. O. V. Belyakov, K. M. Prise, K. R. Trott and B. D. Michael, Delayed lethality, apoptosis and micronucleus formation in human fibroblasts irradiated with X-rays or α-particles. *Int. J. Radiat. Biol*. **75**, 985 – 993 (1999).

56. J. Rickards and E. P. Zironi, Chlorine loss from polyvinyl chloride under proton bombardment. *Nucl. Instrum. Methods B* **56/57**, 687 – 689 (1991).

57. B. J. Kirby and G. J. F. Legge, Ion beam induced damage and element loss during a microanalysis of biological tissue. *Nucl. Instrum. Methods B* **54**, 98 – 100 (1991).

58. M. Hacke, J. Meijer, A. Stephan, H. H. Bukow, M. Hofert and C. Rolfs, Radiation damage of mylar foils by a nuclear microprobe. *Zeitschrift für Physik A* **346**, 309 – 318 (1993).

59. R. Buchalla, Radiolysis products in gamma-irradiated plastics by thermal desorption – gas chromatography – mass spectrometry. Ph.D. Thesis, Technical University of Berlin, 2000.

60. C. F. Bersch, R. R. Stromberg and B. G. Achhammer, Effect of radiation on plastic films. *Modern Packaging*. **32**, 117 – 121 (1959).

61. K. Cwiek-Ludwicka, Polyethylene terephthalate (PET) – Health aspects and food packaging application. *Rocz. Panstw. Zakl. Hig.*. **54**, 175-182 (2003).

62. J. M. Nelson, A. L. Brooks, N. F. Metting, M. A. Khan, R. L. Bushbom, A. Duncan, R. Miick and L. A. Braby, Clastogenic effects of defined numbers of 3.2 MeV alpha particles on individual mammalian cells. *Radiat. Res.* **145**, 568-574 (1996).

63. A. P. Mazzolini, G. J. F. Legge and C. K. Pallaghy, The distribution of trace elements in mature wheat seed using the Melbourne proton microprobe. *Nucl. Instrum. Methods* **191**, 583-589 (1981).

64. F. Watt, G. W. Grime and C. C. Perry, The damage effects of a 1 micron proton beam on a single pollen grain. *Nucl. Instrum. Methods B* **30,** 331 – 336 (1988).

65. P. Van Gansen and N. Van Lerberghe, Potential and limitations of cultivated fibroblasts in the study of senescence in animals. A review on the murine skin fibroblasts system. *Arch. Gerontol. Geriatr*. **7**, 31-74 (1988).

66. B. Praeger, *In-vitro* studies of aging, *Dermatol. Clin*. **4**, 359-369 (1986).

67. C. Keshava, T. Ong and J. Nath, Comparative studies on radiation-induced micronuclei and chromosomal aberrations in V79 cells. *Mutat. Res.* **328**, 63 – 71 (1995).

68. A. H. W. Nias, *An Introduction to Radiobiology*, 2nd ed. John Wiley & Sons Ltd, Chichester, New York, 1998.

69. C. D. Jonah, A short history of the radiation chemistry of water. *Radiat. Res*. **144**, 141 – 147 (1995).

70. T. Nakayama, K. Terazawa and S. Kawakishi, Role of active oxygen species in the toxic effects of glucosone on mammalian cells. *J. Nutr. Sci. Vitaminol*. **38**, 593 – 601 (1992).

71. M. Kaneko, M. Kodama and F. Inoue, Bimodal pattern of killing of Chinese hamster V79 variant cells by hydrogen peroxide. *Free Radic. Res*. **20**, 229 – 239, (1994).

72. D. Roback and J. W. Scrimger, Effects of irradiated medium on growth characteristics of mammalian cells in tissue culture. *Phys. Med. Biol*. **17**, 885 (1972).

73. R. E. Harris and S. M. Pimblott, On ^{3}H β-particle and ^{60}Co γ irradiation of aqueous systems. *Radiat. Res*. **158**, 493 – 504 (2002).

74. T. Moritake, K. Tsuboi, K. Anzai, T. Ozawa, K. Ando and T. Nose, ESR spin trapping of hydroxyl radicals in aqueous solution irradiated with high-LET carbon-ion beams. *Radiat. Res*. **159**, 670 – 675 (2003).

75. HyClone Laboratories Ltd. The development of custom media for individual cell lines. Are media just media?: Part III, *Art to Science in Tissue Culture.* **10**, (1991). Available at http://www.hyclone.com/library/arttoscience.htm

76. P. Rooney and E. G. Wright, Pre-irradiation of tissue culture flasks leads to diminished stem and progenitor cell production in long-term bone marrow cultures. *Cell Prolif*. **26**, 171 – 181 (1993).

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